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Redox processes in pressurised smoked salmon studied by resonance Raman spectroscopy

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ABSTRACT

Non-thermal food preservation technology is based on the application of high pressures up to 600 MPa. Here we report a resonance Raman (RR) spectroscopic analysis of smoked salmon meat after high pressure processing. High quality spectra, which can be obtained even from packed salmon without spectral interference of the packing foil, allow determining pressure-dependent irreversible changes of the main RR-active components of salmon meat, astaxanthin and myoglobin/haemoglobin. High pressure-treatment causes a decrease of the relative RR intensities of astaxanthin as probed with 514 nm excitation which is in line with a slight attenuation of the originally intense red colour of the salmon meat. 413-nm excited RR spectra indicate a heterogeneous broadening of astaxanthin bands accompanied by the formation of deoxy-myoglobin or deoxy-haemoglobin. The results suggest that pressure-treatment facilitates the oxidative degradation of astaxanthin coupled to the reduction of metmyoglobin (methaemoglobin).

1. Introduction

High pressure processing for the improvement of food shelf-life has been known for over one century. It is regarded as a "mild technology" because flavours and vitamins are maintained while pathogenic microorganisms and enzymes can be inactivated (Heinz & Knorr, 2002; Toepfl, Mathys, Heinz, & Knorr, 2006). However, pressurisation may cause unwanted side effects including changes of the colour or texture of meat such as a "bleaching" of red muscle meat from pork, beef, or tuna which is possibly due to denaturation of myoglobin (Cheftel & Culioli, 1997). Such changes have a negative impact on the marketing chances of pressure-treated products since consumers judge the quality of food, *inter alia*, on the basis of the colour. This is also true for smoked salmon, a main product of the Atlantic salmon aquaculture industry. Unlike to pork or tuna meat, haeme proteins are not the main origin of the intense red colour of wild salmon which instead is primarily caused by the carotenoids, mainly astaxanthin and to a smaller extent other carotenoids like canthaxanthin and β-carotene (Andersen, Bertelsen, Christophersen, Ohlen, & Skibsted, 1990). Thus, one may expect that pressure-treatment of smoked salmon may have a qualitatively different and possibly less drastic changes of the visual properties.

The present work aims to elucidate the molecular basis of pressure-induced colour changes of smoked salmon. We have employed resonance Raman (RR) spectroscopy, which selectively probes the vibrational modes of chromophores or protein cofactors by excitation in resonance with an electronic transition. Thus, this technique allows analysing these molecules in complex but optically transparent matrices. In addition, RR spectroscopy is a fast and non-invasive technique which is a prerequisite for in-situ and online analysis (Siebert & Hildebrandt, 2007).

In this work, RR spectroscopy is shown to provide structural information about two key components of smoked salmon flesh, that are astaxanthin and myoglobin (haemoglobin), which can be selectively studied upon appropriate choice of the excitation wavelength. It is shown that the integrity of both components is sensitively affected by pressure-treatment. Specifically, pressurisation promotes the oxidative degradation of astaxanthin coupled to the reduction of metmyoglobin/haemoglobin.

2. Material and methods

Cold smoked salmon chopped and vacuum-packed from the brand "Friedrichs Wildlachs" was purchased from a supermarket. The packing foil consists of polyethylene (65 μm) and biaxial

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oriented polyamide (15 μm). Astaxanthin (purity 92%, Sigma–Aldrich) was dissolved in chloroform (concentration ~1 mM). All experiments were carried out at ambient temperature. UV-vis absorption spectra were recorded on a Unicam UV2 spectrophotometer with a spectral resolution of 0.5 nm, a bandwidth of 4 nm and a scan speed of 600 nm/min. The path length of the measuring cell was 10 mm for all experiments.

Resonance Raman spectra were measured at ambient temperature with the 413-nm excitation line of a Kr^+-laser (Coherent Innova 400) and the 514-nm line of a Ar^+-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 was focused on the solution sample in a rotating cell or onto the smoked salmon flesh sample. The laser power was 0.25–0.4 mW for measurements of astaxanthin and smoked salmon and 3 mW for measuring the Raman spectra of the package foil.

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 from −25 to 100 °C. The volume of the sample holder was 0.75 L. A 1:1 mixture of water and 1,2 propanediol (glycol) was used as a pressure transmitting medium. Vacuum-packed samples were high pressure processed 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). 15 s were needed to reach 600 and 700 MPa. Non-pressurised samples were used as a control.

3. Results and discussion

3.1. Resonance Raman spectra of astaxanthin in solution and in smoked salmon

The main origin for the intense red colour of smoked salmon from aquaculture is the carotenoid astaxanthin. The colour of carotenoids is caused by an allowed π–π^* (S_0–S_2) transition in the visible region of the conjugated polyene system (Fig. 1). For astaxanthin in chloroform this absorption is located at 491 nm such that excitation at 514 nm provides a strong enhancement for totally symmetric vibrational modes of the chromophore (Fig. 2). These are, specifically the C–C and C=C stretching modes of the polyene chain which, for astaxanthin in chloroform, are observed at 1157 cm^{-1} and 1518 cm^{-1}, respectively. The position of the former band remains unchanged upon 413-nm excitation and is, within the experimental error, the same as that previously observed (Merlin, Thomas, Shone, & Britton, 1987) for 488-nm excitation. The 1518-cm^{-1} band, however, evidently varies with the excitation lines since its peak position shifts up to 1521 cm^{-1} for 413-nm excitation (spectrum not shown). The slightly higher frequency at 1523 cm^{-1} reported by Merlin et al. (1987) is attributed to both the different excitation line (488 nm) and the protic solvent (methanol) used in that study. These frequency variations with the excitation line suggest that the band at ca. 1520 cm^{-1} is actually composed of two closely spaced vibrational modes that display different excitation profiles. This interpretation is in fact confirmed by theoretical and experimental studies on carotenoids that are currently under way (Tschirner et al., 2008).

The RR spectra of astaxanthin in chloroform and smoked salmon flesh are similar except for C–C stretching mode which shifts down from 1157 cm^{-1} in chloroform to 1155 cm^{-1} in salmon (Fig. 2). In salmon, most of the astaxanthin molecules are assumed to be bound to actinin approximately in a 1:1 ratio whereas a minor fraction might also be intercalated in lipid bilayers (Matthews, Ross, Lall, & Gill, 2006). The spectrin-like repeats of α-actinin constitute an axially curved and twisted rod-shaped domain which is believed to provide high affinity binding sites for hydrophobic ligands such as carotenoids (Otey & Carpen, 2004). Thus, hydrophobic interactions with α-actinin are likely to cause the small frequency shift of the C–C stretching mode of astaxanthin, possibly due to subtle changes of dihedral angles of the polyene chain (Merlin et al., 1987). Other carotenoids (e.g. canthaxanthin) or astaxanthin stereoisomers are only present in salmon in low concentrations and thus are not likely be the origin for the observed frequency shift.

Due to the strong resonance enhancement, it is possible to selectively probe the RR spectrum of astaxanthin in packed smoked salmon without interference by non-resonant Raman bands of the optical transparent packing material (Fig. 3). Using the confocal microscope, the laser beam was focused directly on

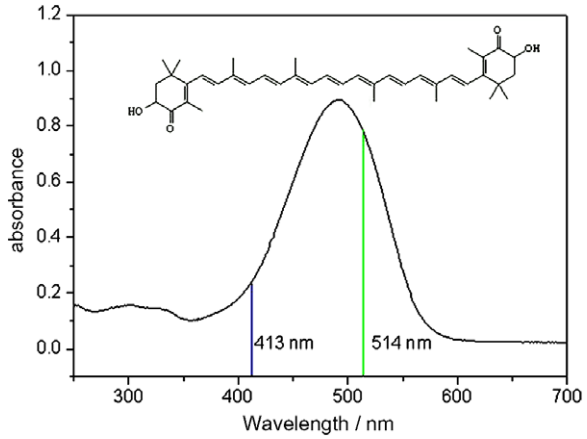

Fig. 1. Chemical structure and absorption spectrum of astaxanthin in chloroform. The laser lines used for RR (413 nm and 514 nm) are indicated.

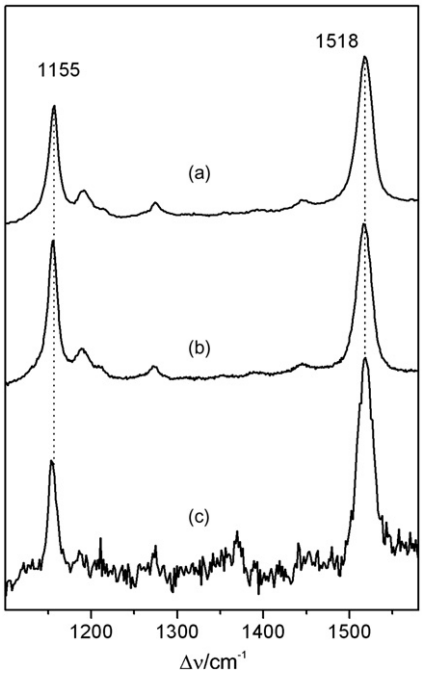

Fig. 2. RR spectra of: (a) astaxanthin in chloroform excitation wavelength 514 nm; (b) smoked salmon, excitation wavelength 514 nm; (c) smoked salmon, excitation wavelength 413 nm.

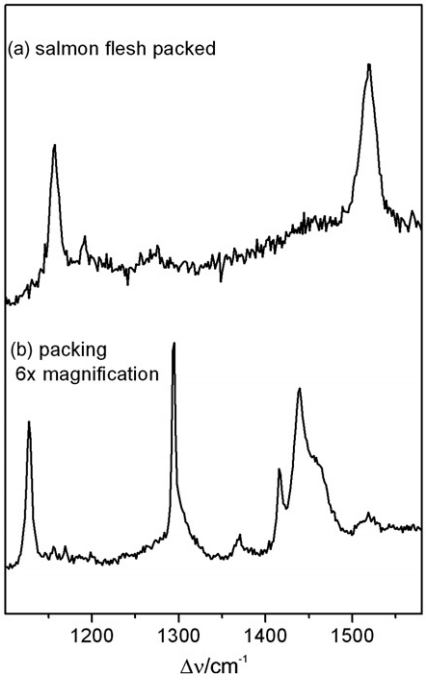

Fig. 3. RR spectra of smoked salmon in retail package (a) and the packing foil (b) with excitation wavelength of 514 nm. Acquisition time and the laser power for the spectrum of the packing material result in a magnification factor of 6.

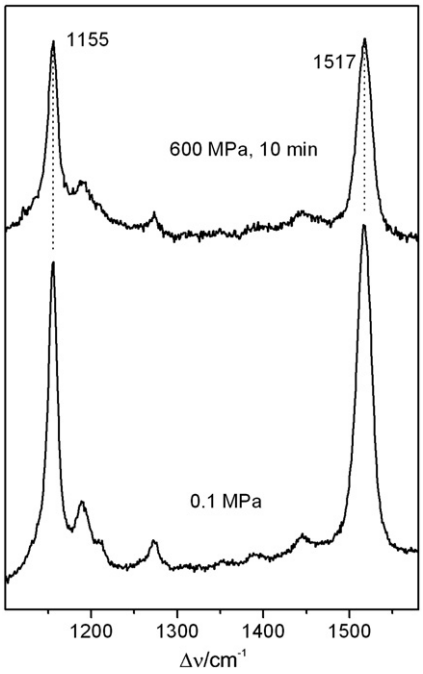

Fig. 4. RR spectrum of smoked salmon after pressurisation (600 MPa, 10 min) and as a reference spectrum non-pressurised smoked salmon (excitation wavelength 514 nm).

the salmon meat surface, thereby significantly reducing the contribution from the packing material. In contrast, an acceptable Raman spectrum only of the packing material, a polyethylene/polyamide foil, requires a substantially higher laser power (3 mW) and longer acquisition time (10 s). Thus, RR spectroscopy can be used for in-situ monitoring of ingredients of smoked salmon and, hence, may become an important analytical and non-destructive tool for food safety and quality.

3.2. Pressure-effects on the resonance Raman spectra of smoked salmon

Visual inspection of smoked salmon flesh, treated at 600 MPa for 10 min, indicate a slight attenuation of the original intense red colour. Upon 514-nm excitation, the RR spectra of such pressurised salmon samples do not display any differences in the positions of the RR bands of astaxanthin as compared to non-pressurised samples. We only note an increased background relative to the RR band intensities (Fig. 4).

Using 413-nm excitation, however, substantial pressure-induced changes are observed in the RR spectrum (Fig. 5). After 600-MPa pressure-treatment the 1520-cm^{-1} peak is distinctly broadened with considerable intensity on the high-frequency side. On the other hand, the C–C stretching of astaxanthin appears to be unchanged within the experimental accuracy of the spectra that, due to the relatively weak resonance enhancement at the edge of the carotenoid absorption band, exhibits only a poor signal-to-noise ratio. Treatment with 700 MPa, however, also leads to a broadening of this band from a width of approximately 10 cm^{-1} to ca. 17 cm^{-1} as compared to non-pressurised sample. The broadening is accompanied by a slight frequency upshift. The underlying modifications of the bound astaxanthin are likely to be associated with a blue-shift of the electronic transition such that bands of the modified astaxanthin are preferentially at 413 nm as compared to 514 nm. This conclusion is consistent with the colour attenuation of the pressure-treated smoked salmon.

Particular noteworthy is the band at ca. 1358-cm^{-1} which in the spectrum of the non-pressurised sample exhibits a very low intensity but a higher frequency at ca. 1370-cm^{-1} (Fig. 5b). The pressure-effect on this band is revealed more clearly by the spectrum obtained from a salmon sample that has been treated at 700 MPa for 1 min. Here we note a substantial intensity increase of the 1358-cm^{-1} band such that is comparably strong to the peaks at ca. 1155 and 1520-cm^{-1} attributable to astaxanthin. The 1358 and 1370-cm^{-1} bands cannot be assigned to astaxanthin but are at a position that is characteristic of the ν_4 mode of a ferrous and ferric haeme protein, respectively (Oellerich, Wackerbarth, & Hildebrandt, 2002). The most likely candidates are myoglobin or haemoglobin which are major protein constituents of salmon flesh. Under physiological conditions, both proteins exist in the reduced state in which the sixth coordination site of the haeme iron is occupied by oxygen (oxy-myoglobin or oxy-haemoglobin) or vacant (deoxy-myoglobin or deoxy-haemoglobin). These forms are characterised by the ν_4 mode at ca. 1355-cm^{-1} and 1376-cm^{-1}, respectively. Whereas the former frequency agrees very well with that observed in the RR spectrum of the pressurised sample, the latter frequency is distinctly higher than that observed for the non-pressurised sample. Despite the poor signal-to-noise ratio of the spectra, the 1370-cm^{-1} peak in the non-pressurised sample can hardly be understood in terms of a superposition of two bands at 1355 and 1376-cm^{-1}. Instead, it may largely result from the ν_4 mode of the ferric haemes in metmyoglobin or methaemoglobin at 1370-cm^{-1}. Note that haeme proteins cannot be the origin for the increased intensity on the high-frequency side of the 1520-cm^{-1} band since haemes do not exhibit bands that have comparable intensity as the ν_4 mode in this frequency region.

On the first sight, these findings are surprising since they suggest a redox process in pressure-treated salmon. Such an effect has not been observed for pressure-treated meat (unpublished results). It is therefore reasonable to assume that the redox reactions involve astaxanthin. In fact, it is known that carotenoids bleach i.e., lose their colour, when exposed to radicals or to

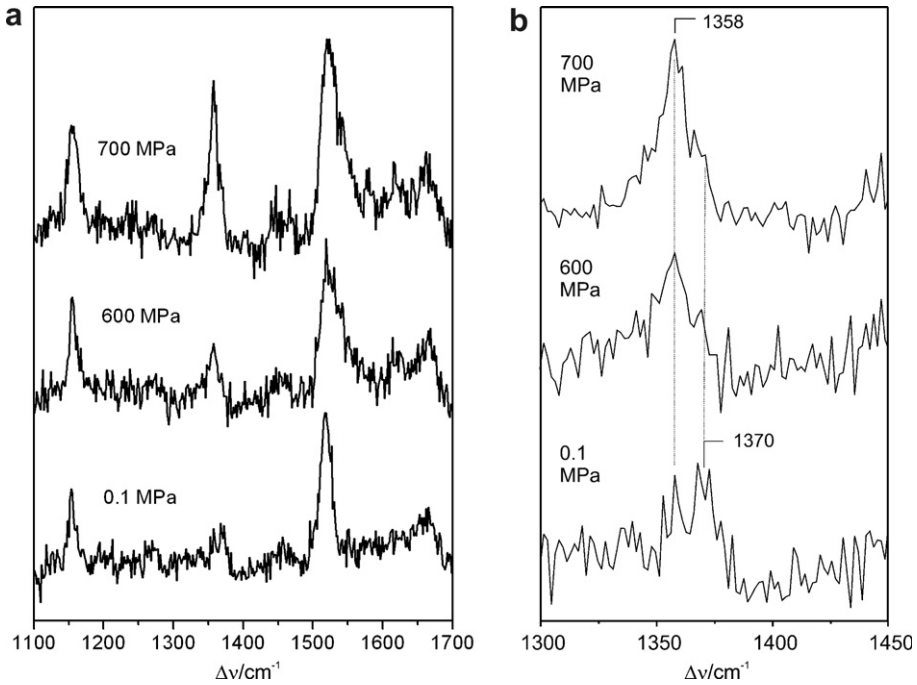

Fig. 5. (a) RR spectrum of smoked salmon after pressurisation (700 MPa and 600 MPa) and as a reference spectrum smoked salmon at ambient pressure (excitation wavelength 413 nm) and (b) enlargement of spectral region.

oxidising species. This process involves degradation of the conjugated double bond system either by cleavage or by addition to one of the double bonds. For example, addition products such as 4-nitro-β-carotene have also been reported for the treatment of carotenoids with smoke containing nitrogen oxides (Krinsky & Johnson, 2005). In any case, the oxidation products are likely to have a lower number of conjugated double bonds than the parent compound. As a consequence, the energy of the first allowed electronic transition and the frequency of the C=C stretching mode are shifted to higher energies (Withnall, Chowdhry, Silver, Edwards, & de Oliveira, 2003). This is indeed in line with the increased RR intensity on the high-frequency side of the 1520-cm^{-1} band (Fig. 5a) and supported by the fact that these features are only enhanced upon 413-nm excitation which evidently provides better resonance conditions for the oxidation products of astaxanthin as compared to 514-nm excitation. The expected smaller effect of astaxanthin oxidation on the C–C stretching is reflected by the less pronounced heterogeneous broadening of the 1155-cm^{-1} band.

At present, we cannot present a conclusive interpretation for the mechanism of oxidation degradation of astaxanthin. Specifically, it remains enigmatic how astaxanthin oxidation is coupled with the reduction of metmyoglobin/methaemoglobin. This coupling is directly reflected by the concomitant intensity increase of the marker bands of ferrous haemes (1358 cm^{-1}) and oxidation products of astaxanthin (>1520 cm^{-1}).

The key question, however, is how pressure-treatment promotes this coupled redox process since oxidation of astaxanthin is not observed in non-pressurised salmon. We propose that pressure-treatment irreversibly affects the binding of astaxanthin to actinin such that the bound astaxanthin becomes accessible to reactive oxidants or that it may be even released from the protein binding site. This interpretation is supported by documented pressure-induced structural changes of myofibrillar proteins (Cheftel & Culioli, 1997 and reference therein). Okamoto and Suzuki (2002) found progressive solubilisation of α-actinin above 200 MPa, indicating that the actinin-astaxanthin complex is affected by high pressure processing. On the other hand it is known that fibrillar protein structures (e.g. collagen) are stabilized by high pressure (Gekko & Fukamizu, 1991). Therefore, it might also be possible that the matrix around the astaxanthin-actinin complex is irreversibly altered due to pressurisation such that astaxanthin becomes accessible to reactive oxidants.

4. Conclusions

The present RR spectroscopic study has shown that high pressure-treatment of smoked salmon opens a reaction pathway for the oxidative attack of astaxanthin, presumably due to a pressure-induced structural change of the host protein, actinin or in the matrix embedding the astaxanthin-actinin complex. The degradation of the carotenoid which is evidently the origin for the colour attenuation of smoked salmon is coupled to the reduction of metmyoglobin/methaemoglobin via a yet unknown mechanism. In a more general sense, this work demonstrates that RR spectroscopy is a powerful tool to gain novel insight into structural changes of essential food ingredients which in turn may be relevant for developing and optimising technologies in food processing, preservation and quality control.

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