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Analytical Methods

Quantitative measurement of metmyoglobin in tuna flesh via electron paramagnetic resonance

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ABSTRACT

The potential of electron paramagnetic resonance (EPR) spectroscopy in the quantitative determination of metmyoglobin (metMb) in tuna flesh was examined and compared with conventional visible spectrophotometry (VIS). Both fresh and stored tuna samples were directly subjected to EPR measurement at −150 °C without pigment extraction, and their metMb concentrations ($[metMb_{TUNA}]_{EPR}$) were determined from a calibration curve. A linear calibration curve with good correlation ($R^2 = 0.987$) was obtained by a plot between EPR intensities and the known $[metMb_{CALI}]_{VIS}$ concentrations, where $[metMb_{CALI}]_{VIS}$ is the concentration of metMb obtained from visible spectrophotometry for the stock metMb solution. The results show that differences between $[metMb_{TUNA}]_{EPR}$ and $[metMb_{TUNA}]_{VIS}$ for tuna meats are negligible at low concentrations of metMb. However, $[metMb_{TUNA}]_{EPR}$ tends to be higher than $[metMb_{TUNA}]_{VIS}$ at a higher concentration of metMb. This is probably due to incomplete pigment extraction from tuna samples that have been stored for a long period of time. This results in experimental inaccuracy of $[metMb_{TUNA}]_{VIS}$ at higher concentrations of metMb. The overall results suggest that the EPR method is a suitable technique for quantitative measurement of metMb in tuna meat without pigment extraction. Since the EPR method operates at −150 °C, this technique could also be very useful in determining the metMb content in frozen tuna meat during low-temperature storage without thawing.

1. Introduction

The colour of tuna meat is an extremely important characteristic influencing the consumer's purchase decision, especially when it is directly served as sashimi (thinly sliced raw seafood). The red colour of meat depends upon the concentration of myoglobin (Mb) and its derivatives (Faustman, Yin, & Nadeau, 1992; Hood, 1980). During preservation, desirably red tuna meat undergoes discolouration and develops an undesirable brown colour, which results from oxidation of ferrous Mb (deoxymyoglobin, deoMb and oxymyoglobin, oxyMb) derivatives to ferric metmyoglobin (metMb) (Bito, 1965; Bito, 1976). A variety of methodologies of colour measurement has been proposed to observe this discolouration phenomenon in meat extracts; for example, visible spectrophotometry (Bito, 1965; Broumand, Ball, & Stier, 1958; Krzywicki, 1982; Tang, Faustman, & Hoagland, 2004), meat surface via reflectance spectrophotometry (Krzywicki, 1979; Stewart, Zipser, & Watts, 1965) or colorimetry (Ochiai, Chow, & Watabe, 1988).

Both the reflectance spectrophotometry and colorimetry methods are rapid and can reflect colour as well as observation by consumers can (Faustman & Phillips, 2001). However, they are not quantitative determination methods. However, visible spectrophotometry is widely employed to assess meat colour in terms of total Mb concentration and the relative proportions of its derivatives; however, pigment extraction prior to spectrophotometric measurement is necessary. The extraction procedure is cumbersome and destroys the sample (Govindarajan, 1973; Stewart et al., 1965). Additionally, the extraction process may cause production of Mb derivatives not originally present in the meat, for example, by extraction in the presence of oxygen (Dean & Ball, 1960; Stewart et al., 1965), improper filtration (Ehira, Uchiyama, & Kakuda, 1984), using water or high pH buffer as a solvent for extraction (Krzywicki, 1982; Warriss, 1979), and even extraction at high temperature.

Currently, there is a soaring demand for fresh and frozen tuna in the Japanese market and worldwide. The tuna industry has recognised the importance of colour stability to tuna meat marketability. Consequently, tuna is customarily kept in the frozen state immediately after harvest until transportation to local markets to prolong the colour shelf-life of the meat (Chow, Ochiai, & Watabe, 2004; Sasayama, 1984; Zhao, Kolbe, & Craven, 1998). To assess the colour change of fish meat during frozen preservation, thawing is commonly required prior to colour measurements. However, there is a report that the discolouration of red muscles in frozen skipjack and tuna occurs easily during and after thawing (Miki & Nishimoto,

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1987). Therefore, until recently, there has not been a concrete method of colour measurement for frozen tuna completely free from this limitation. Hence, methodologies without pigment extraction and thawing are required to precisely and accurately evaluate the colour change in fresh and frozen fish meat. The precise and accurate evaluation of colour change in fish meat will improve understanding of its mechanism.

Electron paramagnetic resonance (EPR) spectroscopy is one of the useful and powerful spectroscopic methods based on absorption of microwaves by a population of molecules possessing unpaired electrons in the presence of an external magnetic field. Using this method, EPR can easily and independently detect paramagnetic species in the raw material and their environment. EPR has long been used for intensive studies of paramagnetic metalloproteins including metMb (Iizuka & Yonetani, 1970). Typically, EPR studies measure the spin state of the iron atom in haemoproteins. The position of the EPR signal can provide specific information on the iron atom in haem, such as the nature of ligand bindings, symmetry, orientation, and spin state, including the haem environment (Gibson, Ingram, & Schonland, 1958; Ingram & Bennett, 1955). Although other metalloproteins, which are chemical combinations of protein atoms with ions of other metals such as zinc (e.g. DNA polymerase), magnesium (e.g. glucose 6-phosphatase), cupric (e.g. cytochrome oxidase), may exist in ordinary tuna meat, it is found that electron configurations for most of these metal ions differ from that of ferric iron in metMb. The difference in electron configurations results in the difference of the pattern of EPR spectra including the corresponding g values i.e., locations of EPR signals. Therefore, there is no interference from other metalloproteins in the observed EPR signals of ferric iron in metMb. However, the EPR signals of metMb for tissue might be interfered from methaemoglobin (metHb), which gives the similar EPR signals to metMb. Since normally haemoglobin is rather lost easily during handling and storage, the primary pigment retained by the intracellular structure is Mb (Livingston & Brown, 1981). As a result, the effect of metHb on the EPR signals of metMb might be negligible. Therefore, the individual information on metMb in tuna meat, which is a heterogeneous complex system, should be efficiently identified from the analysis of the EPR signal. Moreover, such EPR studies in metMb have not required light transmission of the sample and must operate at very low temperatures because the spectra are difficult to analyse at ambient temperature (Iizuka & Yonetani, 1970). These characteristics are interesting for the tuna industry in that frozen fish meat can be directly subjected to EPR measurement without pigment extraction and thawing. EPR measurement is potentially useful for quantitative determination of metMb formation in fish meat during preservation, especially frozen storage.

In this study, we have attempted to examine the potential of the EPR method as a novel method to determine metMb concentration in tuna meat. In addition, we compared metMb concentrations determined by the EPR method and those determined by visible spectrophotometry, which has been employed conventionally.

2. Materials and methods

2.1. Metmyoglobin solution preparation for calibration curve

Equine skeletal Mb (Sigma–Aldrich Co., Tokyo, Japan) was dissolved in 40 mM sodium phosphate buffer (pH 6.8) at 10 mg/ml. To ensure that all Mb compounds were completely converted to Fe^{3+}, 1 mg of potassium ferricyanide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to 1 ml of the prepared stock myoglobin solution. The excess ferricyanide was removed by dialysis against the same buffer. All procedures were carried out at 4 °C.

The stock metMb solution was diluted into several concentrations. The concentration of an aliquot of each dilution was determined according to the method of Warriss (1979) with some modifications. A few micrograms of sodium cyanide (Wako Pure Chemical Industries, Ltd.) were added to each aliquot to convert the pigments to the cyanmet forms, and the aliquot was then centrifuged at 15,000 rpm for 1 h at 4 °C, using a RS-18GL centrifuge (Tomy Seiko Co., Ltd, Tokyo, Japan). The supernatant was subjected to absorbance measurement at 540 nm using a V-630BIO UV–VIS spectrophotometer (Jasco, Tokyo, Japan) and 40 mM phosphate buffer, pH 6.8, as a blank. The metMb concentrations were calculated from the absorbance at 540 nm (A_{540}) using an extinction coefficient of 11.3 mM^{-1} cm^{-1} (Drabkin, 1947) as follows:

$$\text{Metmyoglobin(mM)} = A_{540}/(11.3\ \text{mM}^{-1}\ \text{cm}^{-1} \times 1\ \text{cm}) \quad (1)$$

2.2. Fish sample preparation

Fresh bluefin (*Thunnus thynnus*) and bigeye (*T. obesus*) tuna were purchased as raw fillets from a local fish retailer. For the bluefin tuna sample, the tuna fillet was obtained from a single portion of ordinary dorsal muscle. For the bigeye tuna, the samples were taken from various tuna fillets of ordinary dorsal muscle. Tuna fillets were cut into pieces having dimensions of 0.5 × 4 × 6 cm. All samples were wrapped with film made from polyethylene and polypropylene (Mitsui Chemicals Fabro Inc., Tokyo, Japan), and packed individually in zip-lock packs (Asahi Kasei Home Products Corporation, Tokyo, Japan) prior to storage at 5 °C (±1 °C) for approximately 1 week. Fish fillet was randomly chosen for analysis of metMb content with both spectrophotometry and EPR methods at various times. For EPR measurement, a small amount of tuna meat was randomly taken by piercing a plastic cylinder pipe (approximately 3 mm inside diameter) in the fish fillet. The fish sample was then pulled out from the pipe and frozen immediately with liquid nitrogen. This sampling method was done throughout the fish fillet, and the fish samples were kept in a freezer at −90 °C until the determination with EPR method. The residue of fish fillet was minced in a cold mortar (4 °C), and then immediately employed to determine the metMb content with spectrophotometry method.

2.3. Determination of metmyoglobin concentration in fish meat samples with the visible spectrophotometry method

Pigments in fish meat samples were extracted according to the method of Lee, Hendricks, and Cornforth (1999) with some modifications. The minced sample (2 g) was placed into a 50-ml polypropylene centrifuge tube, and 20 ml ice-cold phosphate buffer (pH 6.8, 40 mM) was added. The mixture was homogenised for 10 sec at 10,000 rpm with ART-MICCRA D-8 (ART Moderne Labortechnik, Hugelheim, Germany). The homogenised sample was centrifuged at 8000 rpm for 30 min at 4 °C, using a RS-18GL centrifuge (Tomy Seiko Co., Ltd.). In order to avoid any turbidity of the extracts, the supernatant was filtered with 0.3-μm filter paper (Nihon Milipore Kogyo K.K., Yonezawa, Japan).

Half of the supernatant was subjected to measurement of metMb percentage. The absorption spectra of myoglobin derivatives were determined using a V-630BIO UV–VIS spectrophotometer (Jasco). The spectra were recorded from 350 to 750 nm at the scanning rate of 1000 nm/min using 40 mM phosphate buffer, pH 6.8 as a blank. The percentage of metmyoglobin (%metMb) was calculated with the following equation (Tang et al., 2004):

$$\%\text{metMb} = -0.159R_1 - 0.085R_2 + 1.262R_3 - 0.52 \quad (2)$$

where $R_1 = A_{582}/A_{525}$, $R_2 = A_{557}/A_{525}$, $R_3 = A_{503}/A_{525}$.