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Research Article

Effect of different saturated aldehydes on the changes in sardine (*Sardinella gibbosa*) myoglobin stability

Manat Chaijan
School of Agricultural Technology, Walailak University, Thasala, Nakon Si Thammarat 80160, Thailand

Corresponding author: cmanat@wu.ac.th

Abstract
Effect of saturated aldehydes with different carbon chain lengths, including hexanal, heptanal, octanal, nonanal and decanal, on the changes in sardine (*Sardinella gibbosa*) myoglobin were investigated in a model system at 4°C for 180 min. The formation of metmyoglobin increased with increasing carbon chain length ($p<0.05$). The highest metmyoglobin content corresponded with the lowest oxymyoglobin remaining was found in the presence of decanal ($p<0.05$). The changes in tryptophan fluorescence intensity and soret absorption spectra of myoglobin were observed and varied with aldehydes added. SDS-PAGE revealed that saturated aldehydes did not induce the cross-linking of myoglobin. Generally, decanal was the most reactive saturated aldehyde to alter the fish myoglobin stability.

Keywords: food analysis, fish, seafood, surimi, storage, oxidation, stability, Thailand

Introduction

Residual myoglobin and hemoglobin influence the degree of whiteness of washed fish mince, a critical factor determining surimi gel quality [1]. In general, hemoglobin is lost readily during handling and storage, whereas myoglobin is retained by the intracellular structure [2]. Normally, both heme proteins can be removed during the washing process of fresh fish leading to increased whiteness of the raw material and resulting surimi. However, heme proteins become less soluble as fish muscle quality deteriorates [3]. Lipid oxidation is a major problem in stored fish [4, 5]. Fatty fish may undergo rapid lipid oxidation during refrigerated storage due to their high content of polyunsaturated fatty acids [6]. Chaijan *et al.* [7] reported that both lipolysis and lipid oxidation occurred progressively in sardine (*Sardinella gibbosa*) during 15 days of iced storage. Lipid oxidation generates a wide range of secondary aldehyde products including n-alkanals, trans-2-alkenals, 4-hydroxy-trans-2-alkenals and malonaldehyde [8]. Secondary products from lipid oxidation, especially aldehydes, can induce myofibrillar protein cross-linking, resulting in structural and functional changes in these proteins [9, 10]. Furthermore,

aldehyde-based lipid oxidation products can alter myoglobin redox stability [11]. Covalent modification of equine, bovine, porcine and tuna myoglobins by 4-hydroxynonenal, a product of linoleic acid oxidation, has been demonstrated [12, 13, 14, 15]. In addition, hexenal and hexanal were reported to accelerate the oxidation of tuna oxymyoglobin [15].

The formation of aldehyde lipid oxidation products during post-mortem handling and storage may negatively affect the colour of processed fish muscle. Saturated and unsaturated aldehydes react differently with protein functional groups [16]. Kautiainen [17] reported that saturated aldehydes tended to form Schiff base adducts, while α, β-unsaturated aldehydes formed mixtures of Schiff bases and Michael addition products. Therefore, the objective of this study was to investigate the effect of saturated aldehydes known to result from lipid oxidation on the changes in sardine myoglobin.

Materials and Methods

Fish samples

Sardine (*Sardinella gibbosa*) with an average weight of 55-60g were caught fresh from the Songkhla-Pattani Coast along the Gulf of Thailand. The dark muscle of the fish, off-loaded approximately 12 h after capture, were collected and used for myoglobin extraction.

Extraction and purification of myoglobin from sardine muscle

Extraction and purification of myoglobin was performed according to the methods of Trout and Gutzke [18] with slight modification. The dark muscle (100g) was coarsely minced and mixed with 300ml of cold extracting medium (10mM Tris-HCl, pH 8.0 containing 1mM EDTA and 25g/l Triton X-100). The mixture was homogenized for 1 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). After centrifugation at 9,600*×g* for 10 min at 4°C using the RC-5B plus centrifuge (Sorvall, Norwalk, CT, USA), the supernatant was filtered through a Whatman No. 4 filter paper. The filtrate was then subjected to ammonium sulphate fractionation. The precipitate obtained with 65-100% saturation was dissolved in a minimum volume of cold 5mM Tris-HCl buffer, pH 8.5, which was referred to as the starting buffer. The mixture was then dialyzed against 10 volumes of the same buffer with 10 changes at 4°C. The dialysate was immediately applied to a Sephadex G-75 column (2.6 × 70cm; Amersham Bioscience, Uppsala, Sweden) which was equilibrated with the starting buffer. The separation was conducted using the starting buffer at a flow rate of 0.5ml/min and 3-ml fractions were collected. The fractions containing myoglobin were combined and further purified by ion-exchange chromatography on a HiTrap DEAE-FF column (pre-packed 5ml; Amersham Biosciences, Uppsala, Sweden). After the column was washed with starting buffer, the elution was performed with a linear gradient of 0-0.5M NaCl in starting buffer at a flow rate of 1 ml/min. Fractions of 3ml were collected and those with a high content of myoglobin were pooled.

During purification, the fractions obtained were monitored at 280nm and 540nm using a Shimadzu UV-2100 spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD, USA). The fractions with absorbance at 540nm were pooled and used as "partially purified sardine myoglobin".

Preparation of oxymyoglobin

Oxymyoglobin was prepared by hydrosulphite-mediated reaction of partially purified sardine myoglobin according to the method of Brown and Mebine [19], and the residual hydrosulphite was removed by dialyzing the sample against 10 volumes cold 10mM phosphate buffer, pH 6.5, for 24 h with 5 changes. The concentration of oxymyoglobin in the solution was determined by measuring the absorbance at 525nm [19].

Effect of saturated aldehydes on sardine myoglobin stability

Sardine oxymyoglobin (0.5mg/ml) dissolved in 10mM phosphate buffer, pH 6.5, was mixed with saturated aldehydes with different carbon chain lengths, including hexanal, heptanal, octanal, nonanal and decanal (0.1mM stock in ethanol) at a ratio of 1:1 (v/v). Controls were aldehyde-free, but contained an equal volume of ethanol needed to deliver the aldehyde. After 180min incubation at 4°C, unbound aldehydes were removed by dialysis overnight against 10 volumes of 10mM phosphate, pH 6.5 at 4°C with 5 changes, and the extent of oxymyoglobin oxidation, changes in tryptophan fluorescence intensity and soret absorption spectra and SDS-PAGE pattern of resulting myoglobin were determined as described below.

Determination of oxymyoglobin content

Oxymyoglobin content was determined spectrophotometrically by scanning from 650 to 450nm with a diffuse-integrating sphere attached to a Shimadzu UV-2100 spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD, USA). The ratio of A_{580} to A_{525} was calculated according to Hansen and Sereika [20]. A large A_{580}/A_{525} ratio indicates a high relative proportion of oxymyoglobin.

Metmyoglobin content

The analysis of metmyoglobin content was performed as described by Lee *et al*. [21]. The sample solution was subjected to absorbance measurement at 700, 572, and 525nm. The percentage of metmyoglobin was calculated using the following equation from Krzywicki [22]:

$$\%\text{Metmyoglobin} = \{1.395 - [(A_{572} - A_{700})/(A_{525} - A_{700})]\} \times 100.$$

Absorption spectra

The absorption spectra of myoglobin solution were taken using a V-530 UV/VIS double beam spectrophotometer (Jasco, Tokyo, Japan) as described by Chaijan *et al*. [23]. The spectra were recorded from 350 to 750nm at the scanning rate of 1,000nm/min using 10 mM phosphate buffer, pH 6.5, as a blank.

Tryptophan fluorescence measurement

Tryptophan fluorescence measurement of myoglobin solutions was performed with a RF-1501 spectrofluorometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 280nm and an emission wavelength of 325nm according to the method of Chanthai *et al*. [24].

Protein patterns by SDS-PAGE

The protein patterns of treated and control myoglobins were visualized by SDS-PAGE under reducing and non-reducing conditions using a 4% stacking gel and 15% separating gel according to Laemmli [25] using a Mini Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). The current for each gel was maintained at 10mA. After separation, the proteins were stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid and de-stained with 40% methanol (v/v) and 7% (v/v) acetic acid. A protein standard consisting of albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa) was used as a reference.

Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests [26]. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 10.0 for Windows, SPSS Inc., Chicago, IL).

Results and Discussion

The effect of different saturated aldedhydes on metmyoglobin formation is presented in Figure 1. When various saturated aldehydes were added into oxymyoglobin solution, metmyoglobin was formed to a greater extent, compared to the control ($p<0.05$). The order of metmyoglobin content as affected by preincubation with saturated aldehydes was decanal > nonanal > octanal > heptanal = hexanal > control. The pro-oxidative behaviour of the saturated aldehydes increased as chain length increased. Hexanal and heptanal seemed to induce the oxygenation of myoglobim as shown by the increase in oxymyoglobin content. This was probably due to the structure stabilization by hexanal and heptanal as evidenced by the similar absorption spectra, as well as the peak height of control, hexanal-treated and heptanal-treated myoglobins (Figure 4). Furthermore, the refolding could occur in hexanal-treated and heptanal-treated myoglobins. However, the increase in carbon chain length from eight to ten carbons caused the oxidation of oxymyoglobin ($p<0.05$; Figure 2). Lynch and Faustman [11] reported that the covalent binding of aldehyde to oxymyoglobin resulted in greater redox instability of oxymyoglobin.

Tryptophan fluorescence intensity of sardine myoglobin with various saturated aldehydes is depicted in Figure 3. Without aldehyde, the heme-globin complex might be compacted and stabilized. In the presence of saturated aldehydes, the increase in fluorescence intensity was found, especially with increasing carbon chain length ($p<0.05$), except for hexanal. The extent of myoglobin unfolding induced by hexanal was greater than those induced by heptanal and octanal. This was probably due to some acceleration mechanism of hexanal on myoglobin unfolding. Hexanal could completely enhance the denaturation of myoglobin and this phenomenon might occur at a higher rate with a higher equilibrium constant. Copeland [27] pointed out that when a protein unfolds, amino acid residues that were buried in the non-polar interior of the protein become exposed to the polar aqueous solvent. Among these residues, tyrosine and tryptophan are commonly found. An intrinsic tryptophan residue in the proteins indicated conformational changes of a tertiary structure

[24]. The fluorescence spectroscopy of this amino acid offers a very powerful tool to monitor the changes in protein structure [27]. Fish myoglobins contained one or two tryptophan residues [24]. Watts *et al*. [28] reported that yellowfin tuna comprised one trytophan residue. From the result, the addition of saturated aldehydes into an aqueous oxymyoglobin system might influence globin conformation as well as apomyoglobin unfolding, resulting in the increase in fluorescence intensity.

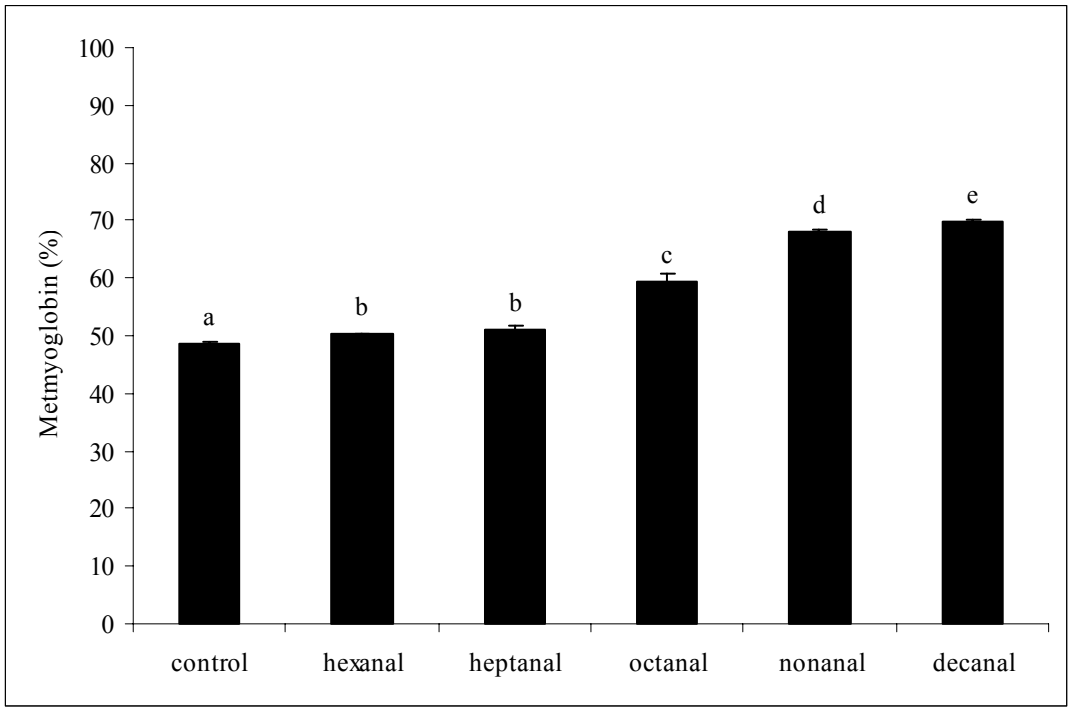


Figure 1. Metmyoglobin formation of sardine myoglobin incubated with various saturated aldehydes at 4°C for 180 min. Bars represent the standard deviation from triplicate determinations. Different letters indicate significant differences ($p<0.05$).

Effects of saturated aldehydes on the absorption spectra of sardine myoglobin are shown in Figure 4. The strong absorption of myoglobin was located in the blue region (350-450 nm) or soret band (Figure 4). The soret peak of both treated and control myoglobin was found at 405nm. Similar peak height was observed among control, hexanal-treated and heptanal-treated myoglobins, whereas myoblobin added with decanal showed the highest peak (Figure 4). From the result, the addition of saturated aldehydes caused the changes in absorption characteristics of myoglobin as evidenced by the changes of the absorbance in the soret region. The higher peak height tended to increase with increasing carbon chain length. In this case, it can be postulated that the conformational changes of sardine myoglobin was induced by increasing aldehyde chain length. Millar *et al*. [29] reported that the spectra of myoglobin vary considerably depending on the state of iron and the molecule to which it is bound.

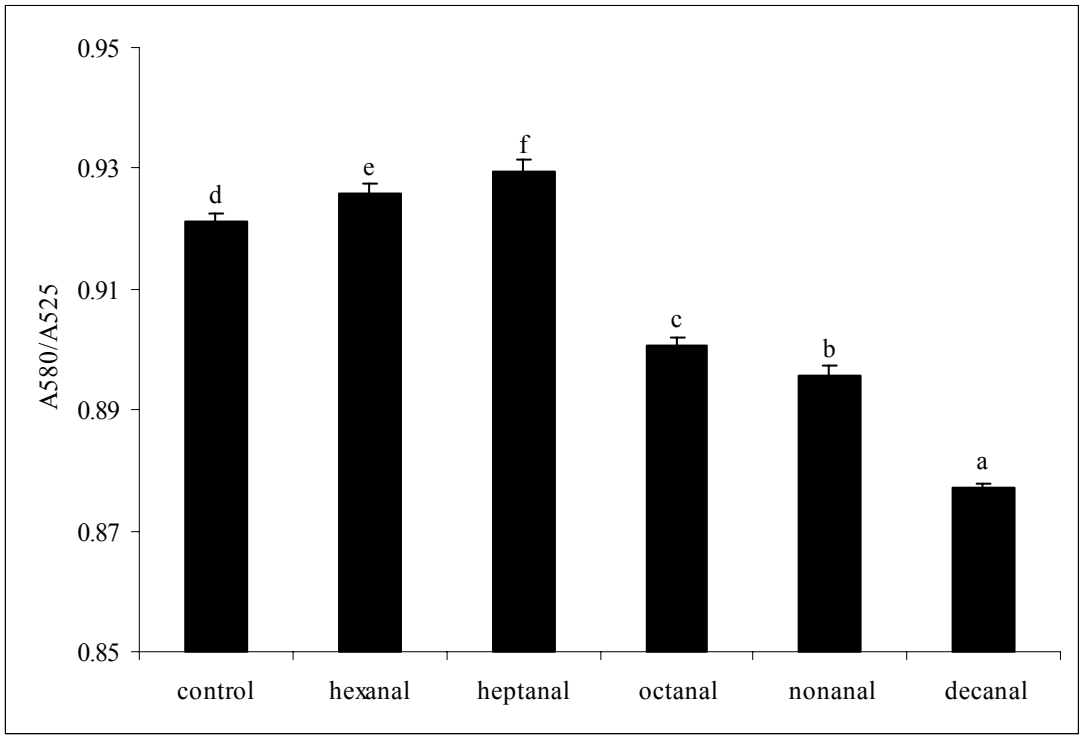


Figure 2. Oxymyoglobin content (A_{580}/A_{525}) of sardine myoglobin incubated with various saturated aldehydes at 4°C for 180 min. Bars represent the standard deviation from triplicate determinations. Different letters indicate significant differences ($p<0.05$).

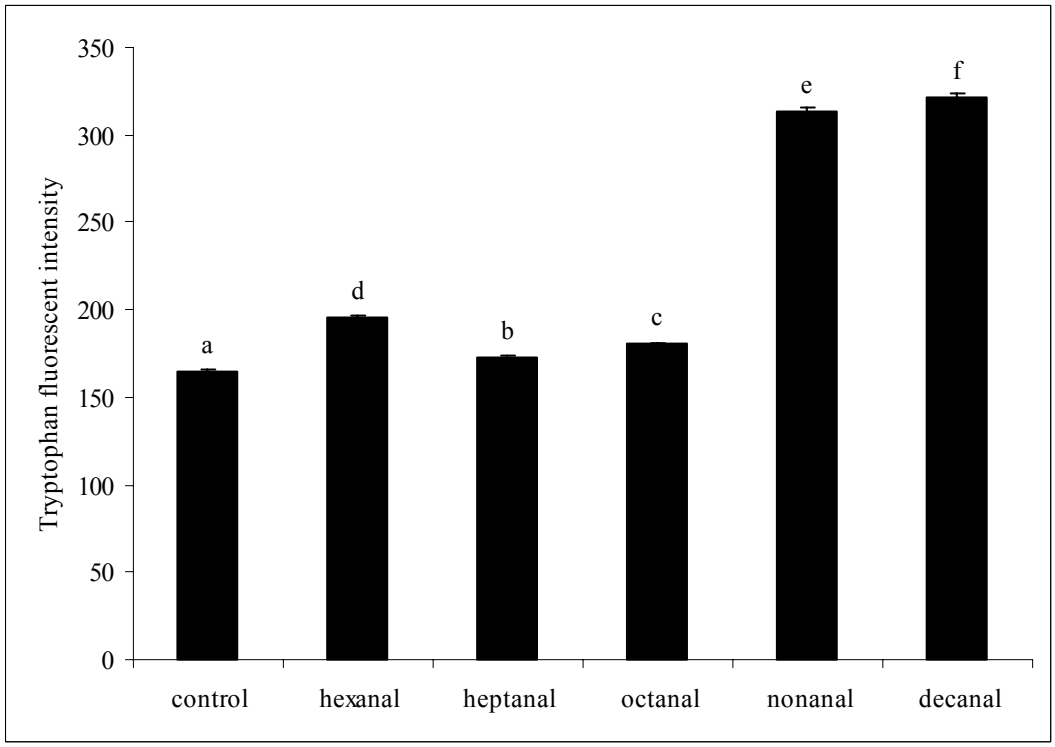


Figure 3. Effect of various saturated aldehydes on the changes of tryptophan fluorescence intensity in sardine myoglobin solution during incubation at 4°C for 180 min. Bars represent the standard deviation from triplicate determinations. Different letters indicate significant differences ($p<0.05$).

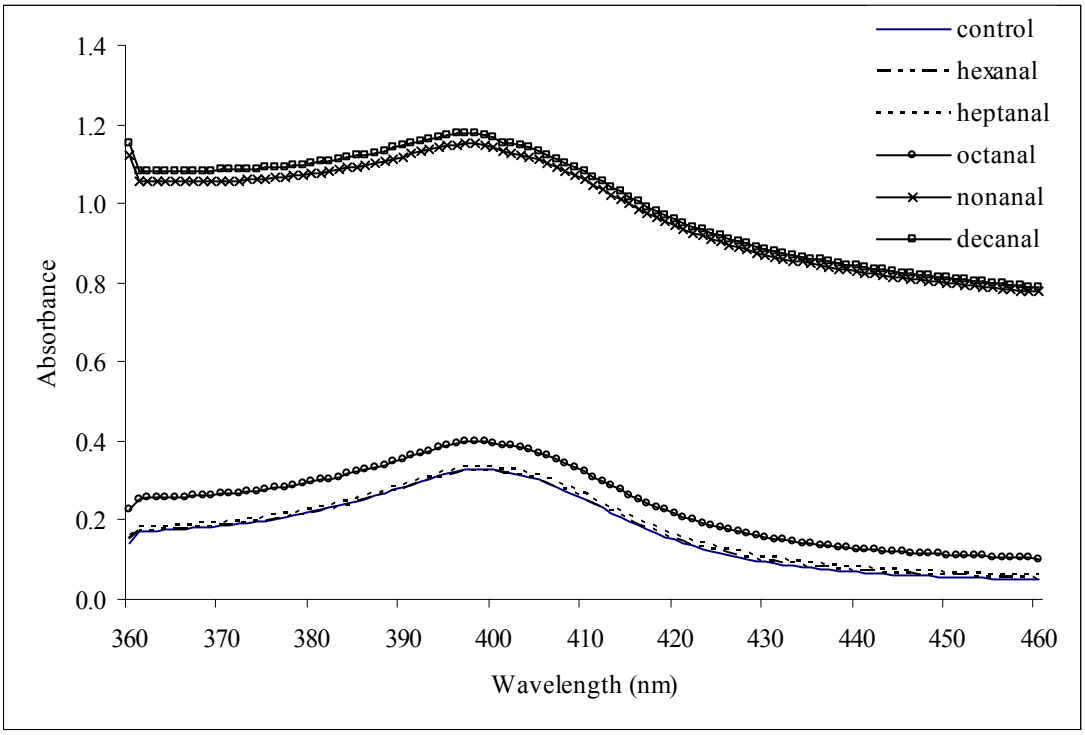
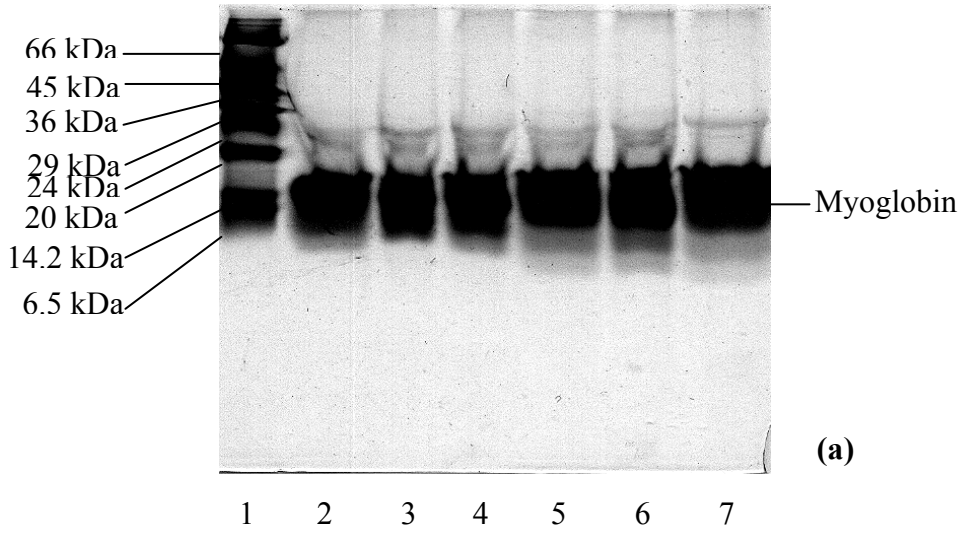


Figure 4. Effect of various saturated aldehydes on the changes of soret absorption spectra of sardine myoglobin during incubation at 4°C for 180 min.

The changes in protein pattern of sardine myoglobin pre-incubated with saturated aldehydes determined using SDS-PAGE are shown in Figure 5. It was noted that under non-reducing condition, there was a band with molecular weight around 28 kDa in all samples including the control (without addition of aldehydes). However, that band was found only in the sample with decanal when analyzed under reducing conditions. Therefore, this protein was most likely the indigenous protein in the myoglobin sample and was stabilized by the disulphide bond, following treatment with decanal. Based on the SDS-PAGE, myoglobin from sardine (major band) did not undergo cross-linking in the presence of aldehyde. Only protein with MW of 28 was changed.



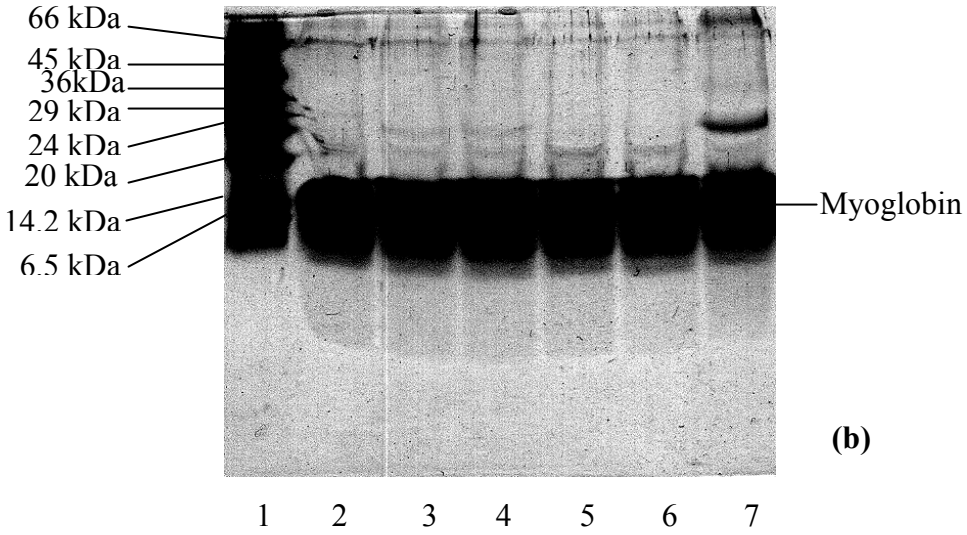


Figure 5. SDS-PAGE pattern of sardine myoglobin incubated with various saturated aldehydes at 4°C for 180 min under non-reducing (a) and reducing (b) condition. 1: Low-molecular-weight marker, 2: control, 3: hexanal, 4: heptanal, 5: octanal, 6: nonanal, 7: decanal.

Conclusion

Saturated aldehydes were able to alter sardine myoglobin by increasing oxymyoglobin oxidation, enhancing the changes in tryptophan fluorescence intensity and soret absorption spectra. Generally, the greater effect was observed with the increasing carbon chain length. Saturated aldehyde did not enhance the cross-linking of myoglobin via disulphide and non-disulphide covalent bonds.

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