FOURIER TRANSFORM RAMAN SPECTROSCOPIC ANALYSIS OF LEAD-EXPOSED MUSCLE TISSUES OF *CATLA CATLA*

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Abstract. Lead is considered to be one of the most common toxic metals present in the environment, exposure to it being a major concern to public health. Fourier Transform (FT) Raman Spectroscopy can provide information on the molecular composition of a substance by detecting and analyzing light that is inelastically scattered from the substance following its excitation by monochromatic laser light. The present study aims to analyze the lead intoxicated muscle tissues of freshwater fingerlings stage of *Catla catla* with FT-Raman spectroscopy. FT-Raman spectra reveal significant differences in absorbance intensities between the control and lead-exposed muscle tissues, reflecting alterations in the biochemical constituents like proteins and lipids. This suggests a decrease in the nutritive value of muscle of *C. catla* fingerlings due to lead intoxication. The amide I maximum observed at ~1665 cm^{-1} indicates that the protein is dominated by α-helical secondary structures. In conclusion, the results suggest that FT-Raman Spectroscopy can be used as a tool for discrimination between normal and metal intoxicated tissues and to detect biochemical changes that occur as a result of metal intoxication.

Key words: Arsenic, FT-Raman, *Catla catla*, muscle, biochemical constituents.

INTRODUCTION

In recent years, increased industrial developments and agricultural processes resulted in increased levels of toxic metals in the environment. The contaminations of freshwater with a wide range of toxic metals are a matter of concern because of the threat to public water supplies and damage caused to aquatic life [2]. Industrial effluents containing heavy metals are routinely released into commercial waterways. Such common practices warrant additional monitoring of anthropogenic agents and their effects on aquatic biota [5]. Fishes are useful organisms in the study of metal contamination, because they explore freely the different trophic levels of the aquatic ecosystem or microbasin.

Received: February 2009;
in final form June 2009.

ROMANIAN J. BIOPHYS., Vol. 19, No. 2, P. 117–125, BUCHAREST, 2009

Lead is considered to be one of the most common toxic metals present in the environment, exposure to it being a major concern to human health. It is used in the preparation of batteries, paints, varnishes and as antiknock compound in gasoline [17]. The toxic effects of lead are known to have an impact on a number of essential body functions, nervous, reproductive and cardiovascular systems [22]. Lead has a high affinity for sulfhydryl groups and mainly inhibits enzymes that contain cystine at their active site and also, indirectly enzymes that use reduced glutathione as cofactor [8]. It binds to body protein and induces damage; the greater the exposure, the more serious the effects [19]. Exposure to lead can result in significant adverse effects to multiple organ systems. It is not accumulated homogeneously within the body. Instead, it circulates throughout the body and is distributed across several physiologically different compartments [1].

Raman spectroscopy, which had earlier been used extensively for investigating pure chemical compounds, has recently emerged in medical research as a tool for characterization of the molecular structure of normal and diseased tissues. Fourier Transform (FT) Raman spectroscopy can provide information on the molecular composition of a substance by detecting and analyzing light that is inelastically scattered from the substance following its excitation by monochromatic laser light. An FT-Raman spectrum is an intrinsic molecular fingerprint of the sample, revealing detailed information about DNA, protein and lipid content as well as macromolecular conformations. In biological samples Raman spectra often exhibit a number of rather sharp bands, compared to infrared spectra which often show broader spectral features [18]. This empirical observation is particularly important in analyzing complex biochemical systems. That is because while infrared spectroscopy is able to yield information about cellular components (eg. proteins, lipids, nucleic acids), Raman spectroscopy gives this information as well as much more information about some of the specific molecules in these groups of components (eg. phenylalanine, tyrosine, and adenine), that is not available from infrared spectra [21]. It is therefore interesting to apply FT-Raman spectroscopy in the present investigation to analyze the lead exposed muscle tissues of freshwater fingerlings stage of *Catla catla*. In the current investigation, the freshwater fingerlings *C. catla* is used, because it is one of the common Indian carps and withstands a wide range of experimental conditions. It occurs in the principal rivers of India and is a moderately fast growing freshwater major carp. In addition, it is of great commercial importance and is renowned for its taste. Muscle is commonly analyzed because it is the main fish part consumed by humans and is implicated as having a part in health risk. Further, muscle is the chief component on which nutritive value of fish may be assessed, and glycogen, protein, and lipid are the main constituents of this tissue

MATERIALS AND METHODS

TEST SPECIES

Healthy *C. catla* fingerlings weighing about 7 ± 1 g body weight and 6 ± 1 cm body length were procured from the Government fish farm, Lalpet, Tamilnadu, India and maintained for a period of 30 days in the general tank and for 3 days in the experimental trough containing dechlorinated tap water for acclimatization to laboratory condition. The physico-chemical parameters of water such as pH, total alkalinity, total hardness, calcium, magnesium and temperature were estimated according to Eaton *et al*. [6] and maintained at optimum level (pH: 7.5 ± 0.3; alkalinity: 212 ± 15 mg/L; hardness: 320 ± 12 mg/L as $CaCO_3$; temperature: 27 ± 2 ºC) throughout the study. Lead stock solution was prepared by dissolving 1.5984 g of $Pb(NO_3)_2$ in 1000 ml of double distilled water. All the working solutions were prepared by diluting the stock solution with distilled water. The 96 h LC_{50} is the basic value in the acute toxicity testing. The calculated 96 h LC_{50} of $Pb(NO_3)_2$ using static bioassay test for *C. catla* is 154.8 ppm of the toxicant [14].

TEST CHEMICALS

The AnalaR Grade lead nitrate purchased from S.D.Fine Chemicals, India, was used without further purification.

EXPERIMENTAL DESIGN

After acclimatization, fish were divided into two groups, each consisting of 20 species, and stocked in 60 liters glass trough of dimension 50 × 40 × 30 cm equipped with continuous air supply. Group I fingerlings were reared in dechlorinated tap water (test water) and treated as control. Fingerlings belonging to group II were exposed to acute concentration (51.6 ppm) of lead for 14 days. All the control and treated fingerlings were fed daily with oilless groundnut cake which had no detectable amount of lead. The test water was changed very 24 hours throughout the experiment by slowly siphoning the water from each container along with waste feed and fecal material. The containers were refilled and redosed with the metal toxicant. Metal analysis of water was carried out periodically and kept with 95% of the required concentration. In the sub-acute toxicity study, no mortality was found in any group throughout the treatment period. At the end of the experimental periods the fish were dissected using plastic materials; muscle organs were separated and stored at –80 ºC until spectroscopic studies were carried out. The samples were lyophilized and made into a fine powder.

SPECTROSCOPICAL ANALYSIS

The FT-Raman spectrum of muscle tissues of *C. catla* fingerlings were recorded at IIT, Chennai, India in the region 3500–250 cm^{-1} using Bruker (Karlsruhe, Germany) IFS 66 interferometer equipped with an FRA 106 module. The 1064 nm line at 300 MW from a continuous wave Nd: YAG laser was the excitation source. Typically 250 scans with a scanning speed of 200 cm^{-1} min^{-1} at 4 cm^{-1} spectral resolution were collected from each sample. The spectral range of the acquired spectra was from 3500 to 250 cm^{-1}.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 11.5 software. The differences between the corresponding values between the control and intoxicated groups were established by the Student's t-test. A probability level (p-value) of less than 0.05 is regarded as statistically significant.

RESULTS

Raman spectroscopy is a non-destructive technique and can provide quantitative chemical composition and identify tissue constituents. A stack plot of the FT-Raman spectra of the control and lead-intoxicated muscle tissues of *C. catla* fingerlings in the region 3500–250 cm^{-1} are presented in Fig. 1. The Raman spectra are typical of those obtained from biological tissues. The spectra are apparently very similar except reduction in intensity of absorption of bands between the control and lead-exposed tissues. These changes in absorption of specific vibrational bands suggest changes in the relative concentrations of proteins and lipids of the muscle tissues due to lead toxicity. The tentative biochemical assignments of individual peaks are given in Table 1. The detailed spectral analyses were performed in three distinct wave number regions, namely 3500–2800 cm^{-1}, 1800–1500 cm^{-1} and 1500–250 cm^{-1}, since identifiable Raman bands were observed mainly in these regions only. The assignment of the Raman bands of proteins, lipids and nuclei acids is based on the available literature values.

Lipids give rise to number of absorptions in Raman spectra. The most intense of these absorptions are found in the 3500–2800 cm^{-1} region, attributed to asymmetric and symmetric stretching vibrations of CH_3 and CH_2 groups of acyl chains. The most intense band observed at 2934 cm^{-1} in the control and lead-exposed muscle tissues has been assigned as C–H stretching of lipids and proteins [21].

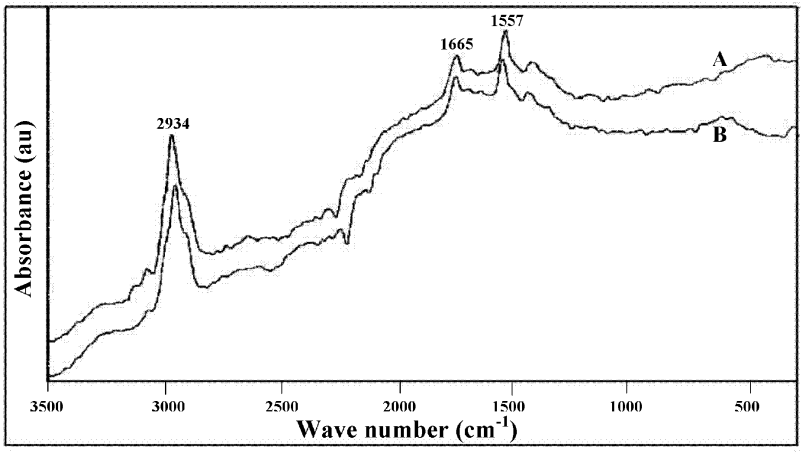

Fig. 1. Stack plot of the FT-Raman spectra of the control (A) and lead-intoxicated (B) muscle tissues of *Catla calta* fingerlings.

Table 1

Vibrational assignment of FT-Raman spectra of the control and lead exposed muscle tissues of *Catla catla* fingerlings

Wave number (cm^{-1})		**Peak assignments**	**Ref.**
Control	**Lead Exposed**		
2934(s)	2934(s)	C–H stretching of lipids and proteins	[21]
1665(m)	1667(m)	Inplane C=O stretching of proteins (Amide I) (α-helix)	[7,10, 13,16, 21]
1612(w)	1605(w)	C=C bending of Phenylalanine, Tyrosine, CCH aromatic amino acids of proteins	[10,13]
1557(s)	1556(s)	C–N stretching / N–H bending of proteins (Amide II)	[7,16]
1456(m)	1456(m)	CH_2 symmetric bending of proteins and lipids	[10,15]
1346(w)	1347(w)	CH_2 deformation (Amide III)	[7]
1131(w)	1131(vw)	C–C, C–N stretching of proteins	[18,23]
1092(w)	1092(w)	PO_2^- symmetric stretching vibrations of the DNA back bone	[10,13, 21]
1013(vw)	1008(vw)	υ (CC) skeletal cis conformation	[23]
927(vw)	927(vw)	ρ (CH_3) terminal; C–C stretching (α-helix)	[24]
838(vw)	838(vw)	O–P–O asymmetric stretching of Tyrosine	[13]
614(vw)	614(vw)	C–C twisting of proteins	[13]

s – strong; m – medium; w – weak; vw – very weak

The region 1800–1500 cm^{-1} contains important information of α-helical secondary structure of protein. The medium intensity band observed at ~1665 cm^{-1} and the strong band observed at ~1557 cm^{-1} are assigned respectively to the Amide I and Amide II [4, 7, 12, 16]. The amide I band mainly involves the carbonyl stretching vibrations of the peptide backbone and is a sensitive marker of peptide secondary structure, since the vibrational frequency of each carbonyl bond depends on hydrogen bonding and the interactions between the amide units, both of which are influenced by the secondary structure [23]. The Amide II band arises from amide C–N stretching / N–H bending vibrations of the tissue proteins. The spectral characteristics of both bands are known to be sensitive to protein backbone conformation. The weak band observed at ~1612 cm^{-1} is assigned to C=C bending of Phenylalanine/ Tyrosine and aromatic amino acids of proteins [24].

In the 1500–250 cm^{-1} region, the band observed at 1456 cm^{-1} is mainly due to the CH_2 symmetric bending modes of proteins and lipids. The weak band observed at ~1346 cm^{-1} is assigned to CH_2 deformation and it belongs to amide III [7]. The band at ~1131 cm^{-1} is assigned to C–C, C–N stretching mode of proteins. The weak bands observed at 1092 cm^{-1} and ~1013 cm^{-1} are assigned respectively to PO_2^- symmetric stretching vibrations of the DNA back bone and cis C–C skeletal conformation stretching modes [20]. The very weak band observed at 927 cm^{-1} may be due to the C–C stretching of α-helix conformation of methyl rocking modes [3]. The band at 838 cm^{-1} is assigned to O–P–O asymmetric stretching of Tyrosine. The band at 614 cm^{-1} is assigned to the C–C twisting of protein [3].

An important factor affecting the membrane structure and dynamics is the amount of proteins and lipids in the membranes. Further, the signal intensity and/or the band area give information about the concentration of related fundamental groups. In the present study, the intensity of the C–C band decreased from 3.31 ± 0.06 to 2.90 ± 0.07 due to lead exposure. This suggests that the number of methyl groups in the acyl chains of lipids decreased due to lead exposure. In addition, the intensity of amide I and II bands decreased respectively from 0.75 ± 0.03 to 0.62 ± 0.02 and from 1.14 ± 0.04 to 0.95 ± 0.03. This reduction in the protein content after lead exposure may be due to reduced protein synthesis due to higher affinity of metal compounds towards different amino acid residues of proteins, which is considered as the premier biochemical parameter for early indication of stress.

It is known that physiological and biochemical parameters in fish blood and tissues could change when exposed to heavy metals [3]. Lead, due to their potential toxicity, produces biochemical changes in the muscle tissues of *C. catla* fingerlings, which gives the first indication of stress. During stress, muscle needs sufficient energy, which is supplied from reserve materials, like proteins, lipids and glycogen. This constitutes the vital organic constituents playing an important role in energy metabolism.

Protein is an essential organic constituent in the tissues of animals and plays a vital role in cellular metabolism. Assessment of protein content can be considered a diagnostic tool to determine the physiological phases of the cells, since proteins are very sensitive and early indicators of heavy metal poisoning in biological tissue. As a constituent of cell membrane, protein has a major role in the process of interactions between intra and extra cellular media. In the present study, the exposure of Group II fish to lead caused a significant decline of protein levels ($p < 0.05$) in muscle of *C. catla* fingerlings, which might be due to changes in protein synthesis and/or metabolism. Thus, it is likely that the tissue burden of lead might have caused disturbances in protein metabolism. The decrease in the muscle protein observed in the present study in the lead-intoxicated tissues could be partly due to their utilization in cell repair and tissue organization with the formation of lipoproteins, which are important cellular constituents occurring in cell membranes and cell organelles in cytoplasm. The depletion in protein may result in further modification of enzyme activity (stimulation or inhibition).

Jana and Bandyopadhyaya [11] have also noticed a significant fall in tissue protein levels when *Chanana punctatus* were exposed to lead. Ghosh and Konar [9] have also observed reduction in the protein content of muscle. The reduction in tissue proteins may be due to impaired or low rate of protein synthesis metallic stress or due to their utilization in the formation of mucoproteins, which are eliminated in the form of mucous. The increased protease activity may cause a decrease in tissue proteins. The other reason is to meet energy demands during lead stress mobilization of proteins. Lomte *et al*. [15] have also reported altered protein levels in *Parreysia cylindrica* due to lead toxic stress.

The decrease in the lipid content may be due to increased utilization of lipid to meet additional energy requirements under a stress of low oxygen taken up [13].

Amide I bands originate from vibrations in the peptide bonds of the proteins and reflect the secondary structure of the proteins. In the present study, the amide I peak maximum is found at ~1665 cm^{-1}, which indicates that the majority of proteins in the muscle tissues are in the α-helix conformation [10].

CONCLUSIONS

FT-Raman spectra reveal significant differences in absorbance intensities between the control and lead-exposed muscle tissues, reflecting alterations in the biochemical constituents like proteins and lipids. This suggests a decrease in the nutritive value of muscle of *C. catla* fingerlings due to lead intoxication. The amide I maximum observed at ~1665 cm^{-1} indicates that the protein is dominated by α-helical secondary structures. In conclusion, the results suggest that FT-Raman Spectroscopy can be used as a tool for discrimination between normal and metal intoxicated tissues and to detect biochemical changes that occur as a result of metal intoxication.

Acknowledgements. Authors would like to express their sincere thanks to the authorities of Annamalai University for providing all necessary laboratory facilities.

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