Use of Fourier Transform Infrared Spectroscopy to Study Cadmium-Induced Changes in Strongylocentrotus nudus gonad

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Abstract: In this paper, the effects of 0.1 and 0.01 mM cadmium on *Strongylocentrotus nudus* gonad were studied by means of fourier transform infrared spectroscopy. The second derivative spectra and curve-fitting analysis revealed adverse effects of cadmium stress on the metabolism of lipids and proteins in gonad. For lipids, cadmium treatment shifted bands position to higher wavenumer and decreased bands area, especially those bands contributed from CH_2 stretching vibration group, suggesting that the structure of lipids constituents were disordered and the contents decreased. Taken account to proteins, cadmium resulted in increasing fraction of β sheet structure and decreasing fraction of α helix, as well as declining protein contents. Compared with 0.01 mM treatment, the higher concentration showed more significant effects, such as bands position shifting to a larger extent and formation of 1692 cm^{-1} band within Amide I regions. The results demonstrated that FTIR spectroscopy is a promising tool for detection of cadmium induced changes in the context of molecular structure observation in urchin gonad.
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1. Introduction

Following the development of metal-working industries and the extensive use of heavy metal- containing chemical fertilizer, the problem of heavy metal pollution on environment has emerged and became increasingly attractive (Soualili et al., 2008). In fact, there are growing studies about the heavy metal pollutants in the marine environment and on the accumulation in tissues of various organisms (D'Souza et al., 2008; Das, 2000; Dumas and Miller, 2003; Esslemont et al., 2003; Fernandez and Beiras, 2001). Among the three most polluting heavy metals, cadmium (Cd) is thought to be the most toxic to organisms and poses the greatest threat to the environment (D'Souza et al., 2008; Henczová et al., 2006).

As being sensitive to heavy metal stress, different sea urchin species have been used to sensor seawater pollutant and to elucidate the mechanism of heavy metal toxicity (Agnello et al., 2007; Garman et al., 1997; Kobayashi and Okamura, 2004; Nacci et al., 2000; Quiniou et al., 1999; Roccheri et al., 2002; Soualili et al., 2008; Soualili and Guillou, 2009). Experimental evidence suggests that exposure to Cd may cause a cascade of events including generation of reactive oxygen species (Howlett and Avery, 1997; Waisberg et al., 2003), depletion of glutathione (Shimizu et al., 1997), inhibition of enzymes involving in DNA synthesis and repair (Giaginis et al, 2006), and DNA single-strand breaks (Schröder et al., 1999), eventually resulting in cellular damages and genetic mutation (Henczová et al., 2004; Waisberg et al., 2003) .

Although numerous studies were performed on sea urchin under Cd stress by means of biochemical and genetic methods, the majority of research focused on sperm, embryo and larvae rather than the gonad tissue, which serves as edible and reproductive tissue where the sperm and egg cells are generated. In fact, this organ, due to its large volume and its function of gametogenesis, is a prime target for heavy metal stress. Therefore, the Cd induced damage will adversely affect the physiological activity. It has known that Cd could interfere on gametogenesis, inhibit embryogenesis, induce cell apoptosis, and interrupt development at different stages (Agnello et al., 2007, Au et al., 2001). However, these phenomena are most likely of results of cadmium stress instead of the initial events to trigger the following results on exposure to Cd. Unlike conventional methods, Fourier transform infrared spectrometry (FTIR) is a convenient and easier handing method to detect the conformational changing of biological molecular components and these conformational disordering as well as subsequent disturbed intramolecular or intermolecular interaction is the key for inducing malfunction.

The vibration of chemical bond absorbs radiation in the IR region between 4,000 and 400 cm^{-1}. Each functional group in a molecule has characteristic absorption frequencies in the IR spectrum. Particularly, FTIR can be used to probe the structure of biological composition or its chemical group, and the composition and structure of molecular functional group can be determined by

analyzing the position, width and intensity of acquired spectra in a complex biological system when rely on certain algorithms (Corte et al., 2010; Yee et al., 2004).

The advantages of FTIR method enable it to examine the initial response to stress with high sensitivity through acquire rapidly spectra from very small amounts of samples (Corte et al., 2010), and offers a fast method to fingerprint the global cellular features under specific conditions (Alvarez-Ordónez et al., 2010). Compared with traditional physiology methods, the FTIR method has its superiority when analyze certain biological samples, because the FTIR could detect the initial response of cells to biotic and abiotic stimuli even before obviously physiological events were detected, as well as provides structural information on biological molecular thus reflecting the mechanism of damages and adaptive of cells concerning to heavy metal. Consequently, FTIR spectroscopy, together with infrared microscopy, is an important technique to study the cellular changes at molecular level in various biological samples (Alvarez-Ordónez et al., 2010; Cakmak et al., 2006).

In the current study, the effects of Cd treatment on urchin gonad were investigated by monitoring the conformational changing of functional groups of protein and lipids with FTIR spectroscopy. Second derivative spectra and curve-fitting analysis of IR spectrum could acquire accurate data, thus allowing quantitatively analyze these functional groups. The presented results provided novel and essential information on the Cd induced damage on urchin gonad.

2. Material and Methods

Sea urchin *Strongylocentrotus nudus* with approximately 5 cm in diameters were collected in the coast of Dalian (38°54'45" N, 121°36'09" E) of liaoning province, P.R.CHINA, during 15th -17th June, in 2011. The animals were acclimated in laboratory with filtered natural seawater for a week, and then treated with 0.01 or 0.1 mM cadmium chloride solutions for 24h. For control and two treatments, 10 individuals were cultured in three tanks at 22±1 °C, supplied with 50 L O_2-saturated filtered natural sea water. After treatment, the gonads of female urchins were separated from whole body, wash two times with filtered natural seawater, and were freeze dried to remove moisture.

Tablets for FTIR spectroscopy were prepared in Agate mortars, dried samples were ground to fine powders and mixed with KBr (1:100 p/p). Spectra were collected by a FTIR-NEXUSTM (Nicolet, America) instrument. The absorbance spectra were measured between 400 and 4000 cm-1 at room temperature (25±1°C). The background spectra collected under identical conditions were subtracted from the samples automatically. The acquisition parameters were resolution of 4 cm-1 with 32 scans co-added. All spectra were normalized and baseline-corrected with Omnic7 software with default settings, and smoothed with smoothing factor of nine. The spectra for control and Cd treated samples were calculated from 3 repeats and each used a different individual. These replicates were averaged and these averaged spectra for each sample were then used for further data and statistical analysis.

Since the original spectra yield rather broad bands in specific regions, mathematical data treatments such as second-derivative analysis has to be applied to resolve the fine details of interested bands (Wolkers et al., 1998). Second-derivative spectra to determine the position of the band as starting parameters for the curve-fitting procedure were calculated with Omnic 7 software using 11 point Savitsky-Golay algorithm. With curve fitting analysis, one not only resolves the individual component bands, but may also calculate the area from each of the individual bands. Therefore, curve-fitting analysis has become an important tool for qualitative and quantitative analysis of infrared spectra. Curve-fitting of the spectra was performed with the professional PeakFit V4.12 software (http://www.seasolve.com) under its AutoFit Peaks II Second Derivative mode with default settings, thus the precise band position and the area of band were obtained as the results. The functional groups of bands were assigned with Knowitall 7.8 software and referenced other literatures. Statistical analysis was performed using SPSS 11.5 software. Differences were analyzed by the student's *t*-test. A probability level (*p*-value) of less than 0.05 is regarded as significant.

3. Results

Under Cd stress, urchin gonad appeared obviously changing in their color, control gonad is light yellow, whereas Cd treated gonad showed dark yellow, and 0.1 mM Cd has more darker color compared with 0.01 mM Cd treatment. In this research, FTIR spectra were acquired to detect the conformational changes and content variations of the functional groups contributed from protein and lipids of sea urchin gonad due to 0.01 and 0.1 mM Cd stresses, and the average spectra of control and treated samples were showed in Figure 1. Generally, the spectra were complex and consist of several bands arising from the vibration of different groups belonging to proteins, lipids, carbohydrates and nucleic acids, indicating that the gonad enrich in biochemical compositions.

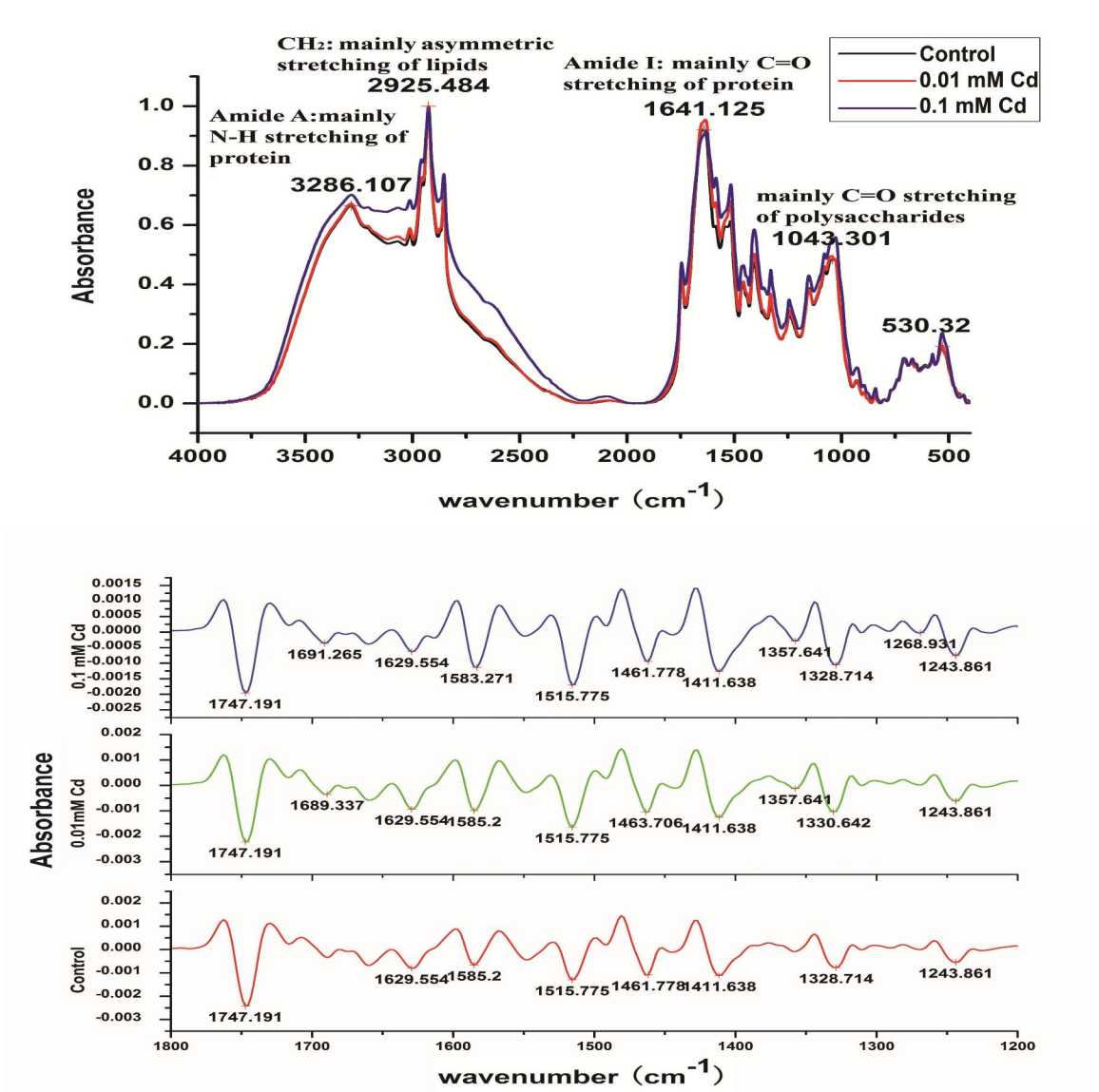

Figure 1. Comparative spectra of control, 0.01 mM Cd and 0.1 mM Cd
Upper: native spectra in the regions of 4000-400 cm^{-1}; Under: the second derivative spectra in the regions of 1800-1200 cm^{-1}. Each curve represents the average spectrum of three individuals.

Accordingly, the majority of dominant bands were fall into two distinct wavenumber regions, namely 3000-2800 cm^{-1} and 1700-800 cm^{-1}, which mainly represented the contribution of groups of lipids, proteins and carbohydrates (D'Souza et al., 2008). In detail, the band located around 3295 cm^{-1} represents N-H stretching vibrations that are mainly caused by proteins (Palaniappan and Vijayasundaram, 2009a; Wolkers et al., 1998). The bands between 3000 and 2800 cm^{-1} mainly represent C-H stretching vibrations that are caused by lipids (D'Souza et al., 2008). The protein absorption bands mainly located between 1700 and 1500 cm^{-1} contained amide I and amide II bands (Warnau et al., 1996), but overlapped with other absorption bands within this region to appeared several prominent bands. The bands between 1500 and 1000 cm^{-1} were of the "fingerprint" region (Palaniappan and Renju, 2009b), amide III and the function group of nucleic acid and carbohydrates contributed to these absorption bands in samples. Overall, the spectrum of control and Cd treated samples differ in the shape of absorbance curve, indicating to obvious changes in structure and contents of biological components due to stress.

The original spectra faced complex multi-component bands which overlapped into a broad unresolved absorption, and the individual component absorptions from condensed phase spectra cannot be

resolved by increasing spectral resolution, causing difficulty in band differentiation and their assignment. Therefore, the second-derivative spectra and curve-fitting processes were performed to distinguish overlapped peaks and achieve more precise calculation on band position and their area. The advantage of this process lies in that the data is de-noised to a great extent and overlapping is largely minimized (D'Souza et al., 2008).

The examination of the second derivative spectra for control and cadmium treated samples revealed several differences (Fig.1), including peak location shifting and peak height variation, especially in the regions from 1500 to 3200 cm^{-1}, indicated that obvious conformational changes were occurred for lipids and proteins. The spectrum of 0.01 mM Cd was more similar to control than 0.1 mM Cd, demonstrated that the higher concentration of cadmium stress induced more obviously spectra changing and significantly affected urchin gonad physiological process. The region 3300 to 3000 cm^{-1} is characteristic for C-H stretching vibrations of C≡C, C=C and Ar-H, while the region from 3000 to 2700 is dominated by the C-H stretching vibrations of -CH_3, CH_2, CH and CHO functional groups respectively (Dumas and Miller 2003; Howlett and Avery, 1997). In this research, the bands within these two regions were considered for analyzing of lipids.

A medium intense band was found around 3012 cm^{-1} in all spectra, which assigned to the vibrating stretching of C=H group belongs to un-saturated lipids (Guillén and Cabo, 1999), and the existing of this peak was well consistent with the conclusion that urchin gonad is rich in several types of un-saturated lipids (Zhu et al., 2010).

The comparative spectra of control with that of 0.01 mM Cd and 0.1 mM Cd showed similar band width and peak positions, likely implying that the composition of gonad un-saturated lipids was kept stable to somewhat. However, the band area of 3012 cm^{-1} of control differ with that of 0.01 mM Cd and 0.1 mM Cd based on curve-fitting analyzing, achieving 0.6992±0.019 for control, 0.6121±0.023 (p<0.001) for 0.01 mM Cd and 0.5031±0.008 (p<0.001)for 0.1 mM Cd. As this band being a useful indicator of the different degrees of un-saturation in acyl chains of phospholipids, the results implied that Cd stress decreased unsaturated lipids contents or reduced the degrees of un-saturation, and the higher concentration of Cd resulted in more obvious effects.

Within 3000-2800 cm^{-1} region, second derivative spectra revealed three prominent bands located at 2961, 2925 and 2852 cm-1 in control, these bands were evident in 0.01 mM Cd and 0.1 mM Cd with wavenumber shifting to a certain extent. However, a shoulder band around 2902 cm^{-1} was found in spectra of control and two treatments, thus PeakFit v4.12 software were used to refine these spectra through curve-fitting analysis. Selecting of this option enable us to find and fit hidden peaks by looking for local minima in smoothed second derivative spectra, and provide more precise fitting results.

After curve-fitting, six, six and eight bands were visible in control, 0.01 mM Cd and 0.1 mM Cd, respectively, suggesting tremendous changes in these regions under Cd stress (Fig.2). Overall, the bands profile of 0.01 mM Cd was more similar to control than 0.1 mM Cd, 6 bands were visible both in control and 0.01 mM Cd with a generally slight up-wavenumber shifting. The dominant bands of control and 0.01 mM Cd at 2958 and 2875 cm^{-1} were assigned to CH_3 stretching vibration, 2925 and 2854 cm^{-1} to CH_2 stretching vibration (Palaniappan and Renju, 2009b), while two weak intensive bands at 2979/2981 cm^{-1} was due to C-H asymmetric stretching vibration. For 0.1 mM Cd, compared with control, two newly emerged weak bands were around 2815 and 2839 cm^{-1} accompanied by 3 bands position shifting, band around 2875 to 2870 cm^{-1}, band of 2895 to 2898 cm^{-1}, and band of 2979 to 2981 cm^{-1}, respectively.

Curve-fitting result showed Cd stress decreased the total area of four dominant bands, from 336.774±3.3336 for control to 36.279±0.7451 (p>0.05) for 0.01 mM Cd, and 29.817±2.3641 (p<0.05) for 0.1 mM Cd. Hence, the qualitatively analyzing of the bands of 3012, 2958, 2925, 2875 and 2854 cm^{-1} confirmed that Cd adversely affected gonad lipid metabolism and the higher concentration resulted in decreasing of lipid content to a larger extent. In addition, the curve-fitting results revealed that cadmium treated with 0.1 mM (0.1 mM Cd) increased the CH_3/CH_2 ratio from 28.81% to 30.28%, which reflected the increased disorder status of lipids. The increased CH_3/CH_2 ratio is also a sign of higher fatty acyl chain unsaturation, which has known to occur with fatty acyl chain peroxidation (Petibois and Deleris, 2004). In the last place, cadmium induced band position to higher value were visible, which suggested that treatment of Cd decreases the fluidity of the lipid acyl chains.

A prominent band located at 2896 cm-1 that belongs to -OCH_3 group stretching vibration (http://www.chem.uni-potsdam.de) was revealed in all spectra. Curve-fitting results demonstrated a weak increasing of band area following the Cd stress was occurring, from 111.469±0.2901 for control to 11.529±0.8506 (p>0.05) for 0.01 mM Cd, and 11.878±0.8429 (p>0.05) for 0.1 mM Cd, also indicating a aggravating of lipid redox status (Wei et al., 2009).

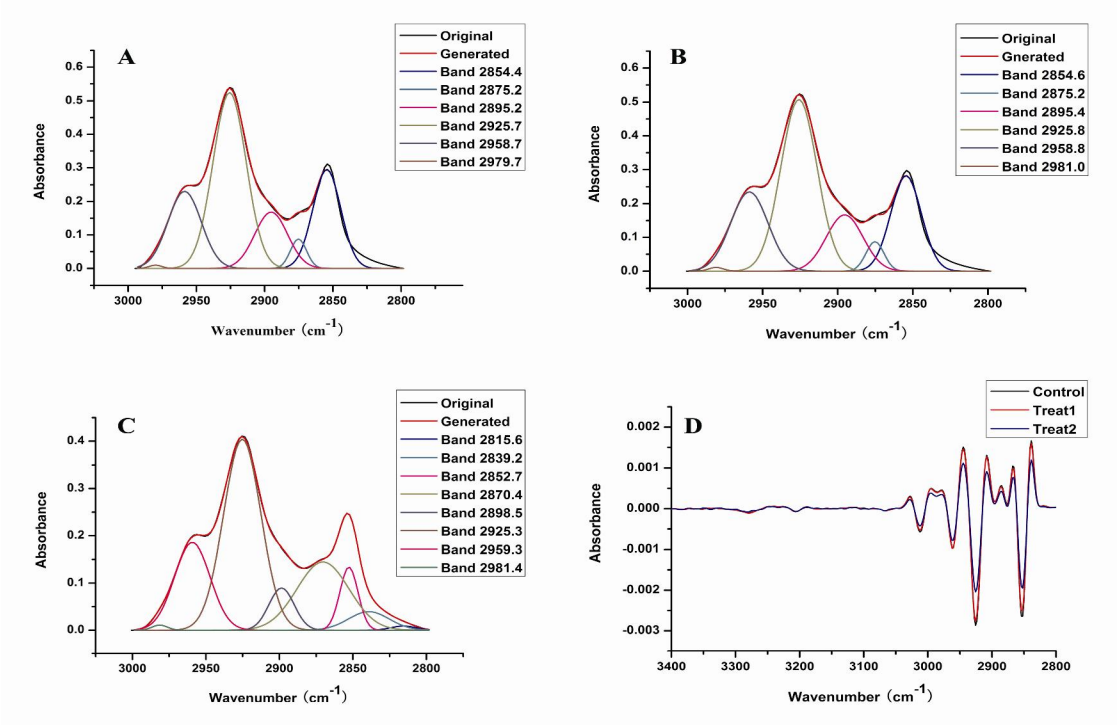

Figure 2. Curve fitting results of regions from 2800 to 3000 cm^{-1}.
The marker "original" represents the raw spectrum after baseline correction; "Generated" represents the overall curve as the results of curve fitting; the last figure represents the comparative second derivative spectra for control, 0.01 mM Cd and 0.1 mM Cd.
A: curve fitting result of Control; B: curve fitting result of 0.01 mM Cd; C: curve fitting result of 0.1 mM Cd; D: second derivative spectra of Control, 0.01 mM Cd and T0.1 mM Cd in the region of 2800 to 3400 cm^{-1}.

Table1. Results of curve fitting of the regions from 1600-1700 cm^{-1}

Control			0.01 mM Cd			0.1 mM Cd			S. A.
B.P.	Area	% Area	B.P.	Area	% Area	B.P.	Area	% Area	
1613	2.02±0.060	8.13	1614	2.06±0.022[d]	8.64	1615	1.75±0.066[a]	9.08	β sheet
1626	4.11±0.258	16.59	1626	4.13±0.278[d]	17.30	1631	4.88±0.480[c]	25.30	β sheet
1638	4.48±0.199	18.06	1638	4.29±0.317[d]	17.98	-	-	-	β sheet
1650	4.54±0.375	18.33	1651	4.40±0.359[d]	18.45	1651	5.56±0.292[b]	28.82	α-helix
1662	4.43±0.241	17.87	1662	4.12±0.191[c]	17.27	1668	3.75±0.106[a]	19.41	Turn
1675	3.26±0.090	13.15	1675	3.04±0.087[b]	12.73	1681	2.36±0.160[a]	12.22	Turn
1687	1.95±0.093	7.88	1687	1.82±0.131[d]	7.64	-	-	-	Turn
-	-	-	-	-	-	1692	0.95±0.041	4.93	β sheet

B.P.: Band position; S.A.: Structure assignment; [a]$p<0.001$; [b]$p<0.01$; [c]$p<0.05$; [d]$p>=0.05$;

Absorption bands around 1746 cm^{-1} correspond to isolated carbonyl group (COOR), indicating ester-containing compounds commonly found in membrane lipid. Lipids make up ~60-65% of a plasma membrane and have a major influence on its properties, thus cadmium stress that disturbs lipids structure or their metabolism would lead to functional disorder of plasma membrane and induced a cascade of downstream events to fulfill its toxic effects on cells. The area changing trend of 1746 cm^{-1} also appeared a constant decreasing profile with increasing of cadmium concentration, from 4.2069±0.1584 for control to 3.9376±0.2022 ($p>0.05$) for 0.01 mM Cd treatment, and 3.4860±0.2238 ($p<0.001$) for 0.1 mM Cd treatment.

The observations suggested that Cd induced changes in the conformational order of lipid acyl chains. It is well known that the CH_2 anti-symmetric and symmetric stretching vibrations give information about the state-of-order of the hydrocarbon tails in

lipids (Akkasa et al., 2007). The sign if it can increase in the frequencies of these bands with Cd treatment indicates that cadmium functions by disordering the lipid system by increasing the number of CH_3 group (Alvarez-Ordónez et al., 2010). Additionally, the characteristic band of CH_2 shift to CH3 demonstrates the oxidative damage of lipid as common phenomena of cells under stress, and this shift is a key event to induce several down-stream events as the reasons to generate cellular damages. Another instance also to proved that Cd stress led to lipid peroxide, the band around 2896 cm-1 appeared an increasing band area with the ascending Cd concentration, indicating that Cd speed up the lipid oxidation. Moreover, Cd stress decreased lipid contents based on 3012, 2961, 2925, 2852 and 1746 cm^{-1} curve-fitting analysis. Taken together, Cd stress resulted in changing of lipids structure of urchin gonad. Since these lipid components could be divided into plasma membrane related and energy metabolism related, thus Cd induced effects on lipids would involve in a wide range of biological processes, which need to be experimental investigated further.

The amide I band of proteins is located in the region of 1700-1600 cm^{-1}. The amide I absorption band is primarily due to the C=O stretching vibration of the amide groups weakly coupled with the in-plane N-H bending and C-N stretching (He et al., 1991). The amide I band of proteins is a complex composite which consists of a number of component bands in terms of α-helices, *β*-sheets (parallel pleated sheet and anti-parallel pleated sheet), turns and random-coil structures (D'Souza et al., 2008). The frequency of the absorption maximum of these bands is conformational sensitive, thus cadmium stress induced shifts of the spectra provided information about structural changes in the proteins of samples.

As described by Palaniappan (2009a; 2009b), the whole Amide I and II area was analyzed by means of curve-fitting in this research. Curve-fitting results uncovered some interesting observations. Firstly, seven bands were found between 1700 and 1600 cm^{-1} for control and 0.01 mM Cd treatment, while only 6 bands for 0.1 mM Cd treatment (Table.1), the absent band in 0.1 mM Cd around 1638 and 1675 cm^{-1} infer to the β sheet and Turn structure of protein, respectively. Secondly, an obvious band position shifting was observed when compared the control with treatment 1- and 2. Giving an example, the band at 1662 cm^{-1} in control was shifted to 1662 cm^{-1} in 0.01 mM Cd treatment and to 1668 cm^{-1} in 0.1 mM Cd treatment. In proteins, the most important hydrogen bonds are those between peptide bonds. Generally, the positions of amide I bands reflect the degree of hydrogen bonding: the higher the wavenumber, the weaker the H bonding (i.e. the less-ordered protein structure). Therefore, these position shifting of amide I bands to higher value are useful indicator of the overall protein disordering under experimental conditions (Yang et al., 2002). Thirdly, a newly emerging band located at 1692 cm-1 only in the spectra of 0.1 mM Cd treatment, together with the increasing area of typical β-sheet (1614 and 1626 cm-1 for control and 0.01 mM Cd treatment, 1615 and 1631 cm-1 for 0.1 mM Cd treatment), likely demonstrated that cadmium stress would induce the protein structure to irreversible protein aggregate (Wolkers et al., 1998).

The result showed the area increased from 37.35 to 40.48 after 0.01 mM Cd treatment with regard to 0.01 mM Cd treatment, but decreased significantly to 31.81 when stressed with 0.1 mM Cd treatment, suggesting a waved variation pattern of the protein content. Similar result reported previously also stated that low dosage of Cd increased protein contents in a short stress period (Ivanina et al., 2008).

Second derivative spectra analyzing in the regions between 3200 and 3400 cm^{-1} uncovered a weak band around 3299 cm^{-1}, which correspond to amide A stretching mode that can generally be associated with N-H and intermolecular O-H molecules (Akkasa et al., 2007). Overall, this band position shifted slightly, being at 3299, 3299 and 3300 cm^{-1} for control, 0.01 mM Cd treatment and 0.1 mM Cd treatment, respectively. Instead, the band area was decreased significantly, from 9.85±0.2654 to 9.68±0.9106 ($p>0.05$) (0.01 mM Cd) and to 6.80±0.3213 ($p<0.001$) (0.1 mM Cd), suggesting a decline of protein contents induced by Cd stress. This observation was well in agreement to the analyzing results for the bands within Amide I and II regions. It was not unexpected results since one of the most popular phenomena of Cd stress is of decreased protein contents.

Considering the changes in Amide I and II regions of control and Cd treated 1 and 2 samples, it could be concluded that the protein synthesis is sensitive to Cd stress in urchin gonad, and the stability of protein secondary structures was able to change after Cd treatment.

Similar to changing profile of lipids, proteins components also appeared decreased bands area and disordered structure. In this paper, Cd induced protein structural change was emphasized analyzed. Several researches with FTIR spectra elucidated that heavy metal could alter protein structure exhibiting up-shifted wavemunbers of bands within Amide I region and increased proportion of β sheet (Henczová et al., 2008). Well consensus with that, obvious bands position shifting to higher value was evidenced, all 6 bands of 0.01 mM Cd treatment were moved generally and the proportion of β sheet was increased accompanied with decreasing of α helix. Unlike 0.01 mM Cd treatment, component bands of 0.1 mM Cd treatment showed more diversity. Except for position shifting to a larger

extent, one band was disappeared and one emerged, which the latter was a good indicator for protein aggregating (Kilimann et al., 2006). Based on the results, it was suggested that under experimental conditions, the gonad proteins were more sensitive than lipids to Cd stress, because the conformational diversity of proteins was more obvious than lipids. It was a reliable hypothesis that Cd could directly bind to proteins with interaction of -SH group of proteins and replace Zn^{2+} which critical for protein maintain the enzymes activity and structure stabling, both results in target protein disorder (Yepiskoposyan et al., 2006). Moreover, Cd induced transcriptomic alternation also generated novel proteins that showed different structures (Georg and Gomes, 2007).

4. Discussions

The analysis of the FTIR spectra collected from urchin gonad treated with Cd revealed that lipids and proteins were sensitive to stress. The results of the current study have provided insight on the Cd induced conformational changes of biomolecular including lipids and proteins, as well as on the content variation of these components. From a physiological aspect, the FTIR analysis construct a direct link between the functional biomolecular and the physiological status under heavy metal stress, because the macromolecular characteristics and their contents are fundamental factors related with several physiological process whereby the cell maintain its normal development and growth. Therefore, in this first study on Cd induced abnormal of lipids and proteins, partial mechanism of Cd stress on urchin gonad were elucidated, and some observations of this paper need biochemical and molecular biological research for further analyzing.

FTIR spectroscopy offers a fast and efficient tool for detection of qualitative and quantitative Cd induced changes in the context of molecular structure level in urchin gonad. In addition, it has the potential as an accurate and sensitive technique for research on heavy metal stress by means of detecting the conformational changing of biological molecular components and these conformational disordering as well as subsequent disturbed intramolecular or intermolecular interaction is the key for inducing malfunction.

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