

FTIR and Proteome Analysis of Sunflower Seedling (*Helianthus annuus* L.) under Lead Stress

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Abstract. The effects of lead stress on the dynamic changes of protein secondary structure and differential protein expression of sunflower seedlings in soil were studied using Fourier transform infrared spectroscopy (FTIR) and two-dimensional (2D) electrophoresis. Under lead stress treatment, the α -helices and β -sheets of protein secondary structure for sunflower seedlings were changed. The content changes, 110% increased in α -helices of roots and 40% declined in β -sheets of leaves, were observed, respectively. Furthermore, 11 differential protein spots were obtained by analyzing 2-DE gel images, while No. 9 were identified by MALDI-TOF/TOF MS, successfully.

1. Introduction

In recent years, metal-ion contamination has become a common problem due to human activity and industrial emissions in farmland soil. Heavy metals are not biodegradable and remain in ecological systems and in the food chain indefinitely, exposing top-level predators to very high levels of pollution. Accumulation of heavy metals in the human body can lead to various toxicological and carcinogenic effects, such as affecting the central nervous system (Hg^{2+} , Pb^{2+} , As^{3+}); the kidneys or liver (Cu^{2+} , Cd^{2+} , Hg^{2+} , Pb^{2+}); or skin, bones, or teeth (Ni^{2+} , Cu^{2+} , Cd^{2+} , Cr^{3+}) [1, 2]. Lead is well known as one of the most toxic metals and is widely distributed in the environment due to its use in batteries, gasoline, and pigments, etc. Lead pollution is a persisting problem and a long-lasting danger to human health and the environment. Even very low levels of lead exposure can cause neurological, reproductive, cardiovascular, and developmental disorders [3]. Therefore, there is a great need for methods of removing heavy metals, especially lead, in the environment and food. Generally, plant cells have a protective system against oxidative stress including enzyme antioxidant system, plants can tolerate low levels of heavy metals in soils, but high levels can cause toxicity symptoms, including growth reduction, leaf rolling and chlorosis. Sunflower has stronger developed root system, drought resistance, barren ability, the tolerance of metal and self-repair ability, etc. Sunflower can accumulate high levels of metal-ions in its tissues under normal growth condition [4].



Up to now, many examples focus on the changes of protein secondary structure of plant seedlings under salt stress by FTIR spectroscopy^[5], or the identification of the inbred lines and hybrids by two-dimensional (2D) electrophoretic technology system^[6]. A few examples of study on sunflower seed protein by 2D electrophoresis have been reported. No examples, namely, using FTIR spectroscopy and 2D electrophoresis to examine protein secondary structure and differential protein of sunflower seedlings under lead stress, have been reported so far. Herein, we design a FTIR and 2D electrophoresis analysis method to study the dynamic changes of protein secondary structure and differential protein expression in sunflower seedlings under different concentrations of lead stress.

2. Material and methods

2.1. Plant material growth

Sunflower seeds (*Helianthus annuus* L.) HA89 were grown and germinated in 20 × 20 cm germination box with 40 seeds per pack. They were soaked in pure water for 8 hours before germination, and then subjected to different treatment groups. The first group was only irrigated with water. The other two groups were irrigated with a solution containing 3.2 mM and 6.4 mM lead acetate, respectively. All groups were irrigated with 4 ml solution every day for a period of 30 days. And the sunflower seeds were germinated and grown at 20°C at night and 25°C on day, respectively. To achieve the high accuracy of the experimental results, three parallel tests were conducted.

2.2. FTIR spectroscopy acquisition of sunflower seedling

The roots, stems and leaves of sunflower seedling, treated with different concentrations of lead stress, were dried immediately at 60°C drying oven for 10 hours. After grinding, the powder samples were mixed with KBr at the ratio of 1:200 and pressed into thin slices for the FTIR transmission measurements. The FTIR spectra were collected using IR200 Fourier infrared spectrometer in the range of 4000-400 cm⁻¹, 32 co-added scans at 2 cm⁻¹ spectral resolution.

2.3. 2D gel electrophoresis

The proteins of sunflower were extracted by the extraction protocols^[7]. Protein concentration was determined using the Bradford method. The detailed procedures are as follows: the protein samples were dissolved in the lysate containing 8 M urea, 4% chaps, 2 M thiourea. For one dimension, the pH gradient strips (IPGS), with precast 18 cm long and pH 3-10, were immersed by a 350 µL rehydration buffer containing 8 M urea, 4% Chaps, 2 M thiourea, 1% ampholytes buffer, 0.001% bromophenol blue, for 12h at room temperature. The second dimension gel was performed at constant current of 10 mA per gel for 25 min and then 30 mA per gel until the bromophenol blue got to the bottom of gel. In order to reduce protein point spread after electrophoresis, 20% TCA solution was used to fix protein for 1h. The 0.15% Coomassie brilliant blue G-250 was used for staining overnight. The gels were scanned using Image Scanner at 300 dpi resolutions and analyzed using ImagemasterTM 2D Platinum software.

2.4. Mass spectrometry analysis

The samples were discolored by 25 mM NH₄HCO₃ and 50% acetonitrile for 30 min, and dehydrated for 30 min by 50% acetonitrile and pure acetonitrile, respectively. Then, the samples were imbibed by the solution containing 25 mM NH₄HCO₃ and 50% acetonitrile at 37°C for 12 h. After that, the samples were acidified by adding the solution containing 5% TFA and 67% acetonitrile at 37°C for 30 min, and centrifuged at 5000 g at 4 °C for 5 min. Finally, the samples were freeze-dried and measured by mass spectrometry.

Protein qualification was acquired using MAILDI-TOF/TOF 4800 (Applied Biosystems, USA) and Nd: YAG laser (355nm) MALDI source with 20 kV. The mass spectra were obtained in range of 700-3500 Da for MS and 40-1050 Da for MS2, respectively. The peptide peak list (*.pkl file) was acquired by combining MS and MS2. Identification of proteins was performed by MASCOT software. And the NCBI nr databank of expressing sequence to retrieve the proteins. Table 1 shows the retrieval parameters.

Tabel 1 Mass spectral databank search parameters

Item	Parameter
Enzyme	Trypsin
Maximum error tolerance	1
Fixed modifications	Carbamidomethylation
Variable	Oxidized
Firstly mass error tolerance	150 ppm
Secondly mass error tolerance	0.6 Da

3. Results and discussion

3.1. The change of lead concentrations on the protein secondary structure

To reveal the change of protein secondary structure in sunflower seedlings under different concentrations of lead stress, the changes of protein secondary structure, especially α -helices and β -sheets, were examined. As shown in Fig. 1, the protein secondary structures of sunflower seedling were β -sheets ($1640\text{--}1610\text{ cm}^{-1}$, $1700\text{--}1691\text{ cm}^{-1}$), random coil ($1650\text{--}1641\text{ cm}^{-1}$), α -helices ($1660\text{--}1651\text{ cm}^{-1}$), β -turns ($1661\text{--}1690\text{ cm}^{-1}$) [8]. The results showed good correlation.

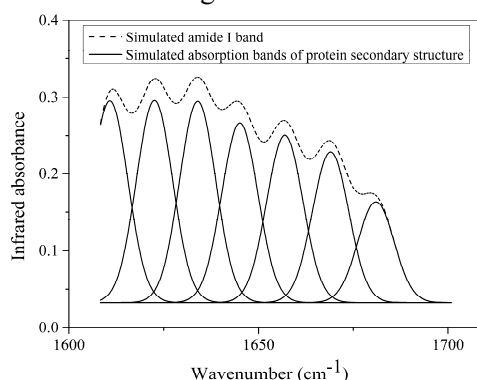
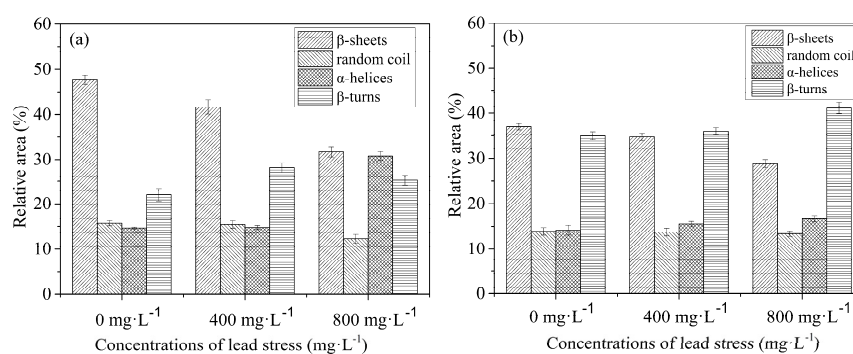


Fig. 1 The protein structure of sunflower seedlings root under lead stress

The content of protein secondary structures, fitting by Gauss, in sunflower seedlings under lead stress were shown in Fig. 2. Comparing with the control roots, stems and leaves of sunflower seedling, the β -sheets and random coil were reduced while α -helices and β -turns were increased. The details of the changes in protein secondary structures were shown in the table 2. It is speculated that the β -sheets and random coil may be transformed into α -helices and β -turns.



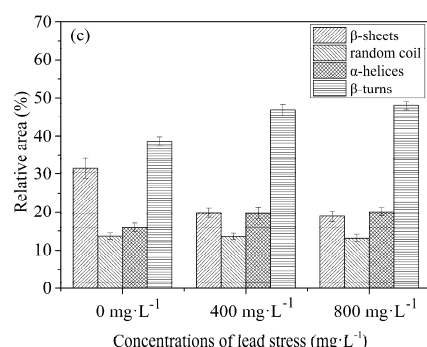


Fig. 2 The relative area under different concentrations of lead stress in sunflower seedling roots (a), stems (b) and leaves (c).

Table 2 The content of changes (+ increase; - reduce) in protein secondary structures of sunflower seedlings roots, stems and leaves under lead stress.

Secondary structures/%	roots		stems		leaves	
	400 mg·L ⁻¹	800 mg·L ⁻¹	400 mg·L ⁻¹	800 mg·L ⁻¹	400 mg·L ⁻¹	800 mg·L ⁻¹
β-sheets/%	-13%	-33%	-6%	-22%	-37%	-40%
random coil/%	-2%	-22%	-1%	-3%	-1%	-4%
α-helices/%	+1%	+110%	+10%	+18%	+24%	+25%
β-turns/%	+28%	+15%	+3%	+18%	+21%	+24%

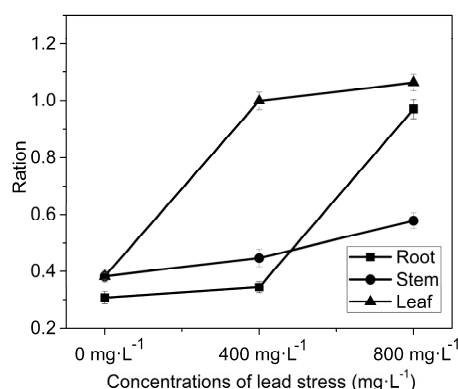


Fig. 3 The ratio of α-helices and β-sheets for sunflower seedlings roots, stems and leaves under lead stress in 0 mg·L⁻¹, 400 mg·L⁻¹, 800 mg·L⁻¹.

The stability of the protein structure has important sense to biological activities. However, the numbers of hydrogen bonding in peptide chain determines the stability of the protein structure. To a certain extent, the ratio of α-helices and β-sheets are directly related to the numbers of hydrogen bonding in protein secondary structure. Therefore, the ratio of α-helices and β-sheets in roots, stems and leaves of sunflower seedling was examined (Fig. 3). The ratio of α-helices and β-sheets were increased in sunflower seedling roots, stems and leaves under different concentrations (400 mg·L⁻¹, and 800 mg·L⁻¹) lead stress^[9]. Therefore, with the increase of the lead concentration, the numbers of hydrogen bonding in peptide chain were increasing and the stability of the protein structure was enhanced.

3.2. The effect of lead concentrations on the protein expression

To confirm differential protein in sunflower seedlings under different concentrations of lead stress from protein level, sunflower seedling proteins under lead stress were separated by 2D electrophoresis and MS/MS (Fig. 4). As shown in Fig. 4, when sunflower seedlings were irrigated with different

concentrations of lead stress ($0 \text{ mg}\cdot\text{L}^{-1}$, $400 \text{ mg}\cdot\text{L}^{-1}$ and $800 \text{ mg}\cdot\text{L}^{-1}$), about 180, 200 and 160 protein spots, respectively, were detected in the 4-10 pH range. The results clearly demonstrate that there were 27 spots displayed in differences in volume by the software matches and 11 protein spots of these spots were significantly differences in expression. 9 spots could be identified based on MASCOT automated retrieval in NCBI database shown in the table 3. And the score higher than 45 and sequence coverage between 59% and 72% were obtained.

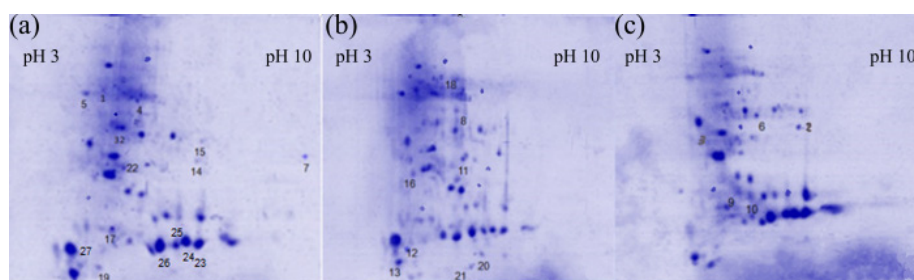


Fig. 4 2D electrophoresis profiles of sunflower seedlings under $0 \text{ mg}\cdot\text{L}^{-1}$ (a), $400 \text{ mg}\cdot\text{L}^{-1}$ (b), $800 \text{ mg}\cdot\text{L}^{-1}$ (c) lead stress

Table 3 Proteins from sunflower seedling identified by MALDI-QTOF/TOF

Protein spot No	Description	Mass
1	Unconventional Myosin [<i>Helianthus annuus</i>]	10115
7	Site-determining protein Two-component system regulatory protein	29733
9	[<i>Pseudomonas syringae</i> pv. <i>helianthi</i>]	21197
10	Ribosomal protein S8 (chloroplast)	15678
15	Ornithine carbamoyltransferase	34022
16	Hypothetical chloroplast RF1 (chloroplast) [<i>Helianthus annuus</i>]	20458
17	TonB system transport protein	14764
25	30S ribosomal protein S20	10006
27	Peroxidase, partial [<i>Helianthus annuus</i>]	10337

The quantities of expressions of protein spots 9 (proteins related to stress resistance) ^[10] and 10 (Chloroplast protein) ^[11] were increased with the increase of the lead concentration. The increase quantities of expressions of protein may be to resist the harm caused by lead stress, which is consistent with the results of Jerusa studies ^[12]. The quantity of expression of protein spot 27 (proteins related to respiration and photosynthesis) was decreased with the increase of the lead concentration. Protein spot 16 showed gradually disappear with the increase of the lead concentration, which had an expression in lead-ion concentration of $400 \text{ mg}\cdot\text{L}^{-1}$, but no expression was observed in lead concentration of $800 \text{ mg}\cdot\text{L}^{-1}$. Protein spot 7 (proteins related to cell division) ^[13] had no expression under lead concentration.

4. Conclusions

In conclusion, the changes of protein secondary structure and differential protein expression of sunflower seedlings, treated with different concentrations of lead stress, were observed by Fourier transform infrared spectroscopy (FTIR) and two-dimensional (2D) electrophoresis. This study was aimed at revealing the physiological mechanism of sunflower seedlings under lead stress.

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