Effects of iron and cupper ions on sulforaphane content and peroxidase activity in *Lepidium draba* seedlings

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Abstract

Sulforaphane is an isothiocyanate which is produced through glucoraphanin hydrolysis via myrosinase activity. In the present study, Sulforaphane content and peroxidase activity has been assessed in *Lepidium draba* seedlings which treated with different concentrations of iron and copper ions for 8 and 16 hours. The results showed that the Sulforaphane content drastically elevated around two-fold compared to control when the seedlings treated with 1 mg/L Fe^{2+} for 8 hours. But, the Sulforaphane content decreased in treated seedlings with higher Fe^{2+} concentrations and any concentrations of Cu^{2+}. Furthermore, promotion of peroxidase activity was observed in the treated seedlings with the both metals. The data revealed that, not only the inhibitory effect of Cu^{2+} on Sulforaphane production was stronger than Fe^{2+}, but its stimulatory effect on peroxidase activity was also remarkable especially after 16 hours treatment. It seems that at low Fe^{2+} concentration reactive oxygen species induced by these metals resulted in inducing of glucoraphanin biosynthesis pathway. While, produced reactive oxygen species in treated seedlings with higher Fe^{2+} concentration and Cu^{2+} led to induce more enzymatic antioxidant system.

Introduction

Sulforaphane (SFN) is an isothiocyanate which can be produced via myrosinase catalyzed hydrolysis of glucoraphanin (Fenwick et al., 1983). Several pharmaceutical effects have been reported for SFN; including anticancer (Grubb et al., 2006; Zhang et al., 2007; Clarke et al., 2008 & Xu et al., 2012), antioxidant (Fahey et al., 1999) and anti-bacterial properties (Fahey et al., 2002).

Glucoraphanin [(4-methylsulfonyl) butyl glucosinolate] belongs to glucosinolates group containing sulfur and nitrogen (Fahey et al., 2001). Up to 2001, about 120 glucosinolates had been identified in plants of the Brassicales order (Fahey et al., 2001). Chemical composition of these metabolites contains a carbohydrate, an amino acid-derived side chain and an aldoximsulfonate moiety (Mithen et al., 2000). Glucosinolatesare discriminated from each other by their side chains originated from amino acids and grouped into aliphatic, aromatic and indolic amino acids (Mithen 2001).

Several plants such as Broccoli (*Brassica oleracea L.*var), Cabbage (*Brassica oleracea*) and Whitetop (*Lepidium draba*) contain large amounts of glucoraphanin (Fahey et al., 2001 & Powell et al., 2005). Myrosinase (β-thioglucosideglucohydrolase, EC 3.2.3.1) catalyzes glucosinolates hydrolysis into glucose and an unstable intermediate called aglycon (Fenwick et al., 1983). Thereafter, the aglycon can be converted into many compounds such as sulfur, thiocyanate, isothiocyanate and nitrile, depending on pH, temperature and presence of different concentration of ions (Uda et al., 1986 & Bones et al., 2006).

L. draba, a noxious weed of the Brassicaceae family (Zargari 1995), can accumulate several heavy metals such as Zn, Cu, Fe, Cd and Ni inside the leaves (Chehregani & Malayeri., 2007). Iron and copper are essential microelements (Taiz et al., 2002). These elements act as cofactor for some enzymes such as

ATP synthase and cytochrome C oxidase; these enzymes play key roles in photosynthesis, respiration, cellular redox systems and nitrogen fixation (Taiz et al., 2002 & Yruela., 2009). However, the toxicity effects of these elements have been established particularly at higher concentrations through inducing oxidative stress (Malekzadeh et al., 2007; Gill et al., 2010 & Sharma et al., 2012). During oxidative stress, reactive oxygen species (ROS) such as superoxide anion, hydroxide radicals and hydrogen peroxide are main radicals that can cause severe damages to the cell membrane, nucleic acids and proteins (Asada 1987). In plants, two defensive mechanisms of non-enzymatic (such as ascorbic acid, glutathione, flavonoid compounds and glucosinolates) and enzymatic (such as catalase, superoxide dismutase and peroxidase) are responsible for sweeping of ROS (Choi et al., 2004).

Despite of *L. draba* that contains only two major types of glucosinolate; glucoraphanin and glucosinalbin (Powell et al., 2005), and suits for easy extraction and purification of SFN precursor (glucoraphanin); most of the studies have been focused on induction of glucosinolates and their breakdown from Brassica species in presence of different elicitors (Kiddle et al., 1994; Smetanska, 2005 & Liang et al., 2006).

In this research, SFN production level was analyzed in *L. draba* seedlings in treatment with different concentrations of iron and copper ions at different time intervals. Furthermore, activity of some key enzymes involved in scavenging of hydrogen peroxide was assayed.

Materials and methods
• Plant growth
Seeds of *L. draba* were collected from around Kerman province (Iran) in the end of May and early June 2012. The seeds were surface-sterilized by immersing in 2% sodium hypochlorite solution for 15 minutes and rinsed several times using sterile distilled water. The seeds were placed on the surface of 1% agar medium in Petri dishes. The plates were placed in a germinator at controlled temperature of $28\pm2^{\circ}C$, relative humidity of 60-65% under photoperiodic condition of 8:16 (dark: light). In this condition about 95% of the seeds germinated and their roots developed. The 7-day-old seedlings were harvested from their medium and washed thoroughly using sterile distilled water and subjected to treatment.

• Elicitor preparation and seedling treatment
Different concentrations of $FeSO_4$ and $CuSO_4$ (0 as a control, 1, 5, 10, 20 and 40 mg/L) were dissolved in distilled water. For each treatment, around 50 seedlings were transferred in 250 mL Erlenmeyer flasks containing 50 mL of the solution. The flasks were shaken at 100 rpm on an orbital shaker at 25°C for 8 and 16 hours. Then, to remove the surface ions, the treated seedlings were rinsed several times with distilled water and immediately frozen in liquid nitrogen, and kept at -80°C until used.

• SFN determination and quantification
SFN extraction and determination was carried out according to the Liang and co-workers (Liang et al., 2006) method with a slight modification. In brief, 1 g of the fresh tissue was grinded with a mortar and pestle and mixed with 1 mL of acidic water. After two hours incubation at $42^{\circ}C$, 5 mL of acetonitrile was added to the mixture, and sonicated for 3 minutes. The resulting mixture was centrifuged at 10000 rpm for 10 minutes at $4^{\circ}C$. Finally, the supernatant was passed through 0.2 μm syringe filter and the SFN content was measured using High Performance Liquid Chromatography (HPLC, Agilent 1100 series, C_{18} column). Mobile phase solvent including acetonitrile/H_2O (65/35 v/v) and a flow rate of 1 mL/min under room temperature, was used in order to separate SFN. In the seedlings, SFN peak was identified by comparing of the retention time with authentic standard (Sigma-Aldrich) that was detected at 254 nm.

• Protein extraction
Protein extraction was done by homogenizing of 0.5 g of fresh tissue in 5 mL of 50 mM potassium phosphate buffer (pH 7.5) containing 1% polyvinyl pyrrolidone, 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 12000 rpm for 15 minutes at $4^{\circ}C$. Protein concentrations in seedling´s crude extracts were determined according to the Bradford method, with bovine serum albumin (BSA) as a standard (Bradford., 1976).

• Antioxidant enzyme assay
Peroxidase (POD) [EC.1.11.1.7] activity was determined as described by Plewa and co-workers protocol (1991). The reaction mixture (3 mL) contained 50 mM potassium phosphate buffer (pH=7), 4% guaiacol, 1% H_2O_2 and appropriate enzyme extract. Activity was expressed as changes in absorbance at 470 nm against mg of protein.

Catalase (CAT) [EC.1.11.1.6] activity was assayed according to the method of Dhindsa (1981). The decline in absorbance at 240 nm was recorded followed by the decomposition of H_2O_2. One unit of catalase is defined as the amount enzyme that decomposes 1 mM H_2O_2 per minute.

- **Statistical analysis**

Experiments were conducted in completely randomized design and each treatment was performed in triplicate.Significance of the treatments was determined by one way analysis of ANOVA variance. Mean value of the data at $p \le 0.05$ was analyzed by Duncan's multiple range tests by the use of SPSS v. 21. The results were expressed as mean values ± standard deviation (SD).

Results

- **The effects of metals on SFN content**

The retention time of SFN was about 4 min after injection into the column as seen for standard SFN and complex of standard - sample (Fig. 1).

SFN content significantly increased in presence of 1 mg/L Fe^{2+} in either time of treatment (8 hours and 16 hours), this increase was more after 8 hours of treatment that it was around 2 fold higher than the control (Fig. 2). Moreover, the SFN content slightly increased at 5 and 10 mg/L after 8 hours of treatment although it was not statically significant. After 16 hours of treatment, SFN content did not changed in treatment with 5mg/L Fe^{2+} concentration in compared to the control (Fig. 2), but at higher concentrations led to decrease in SFN content.
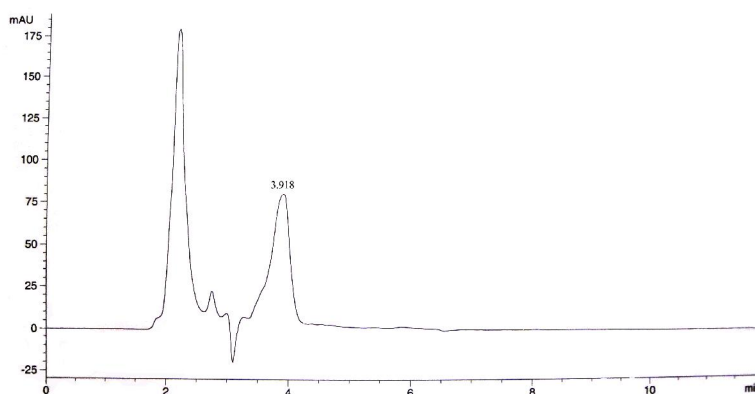

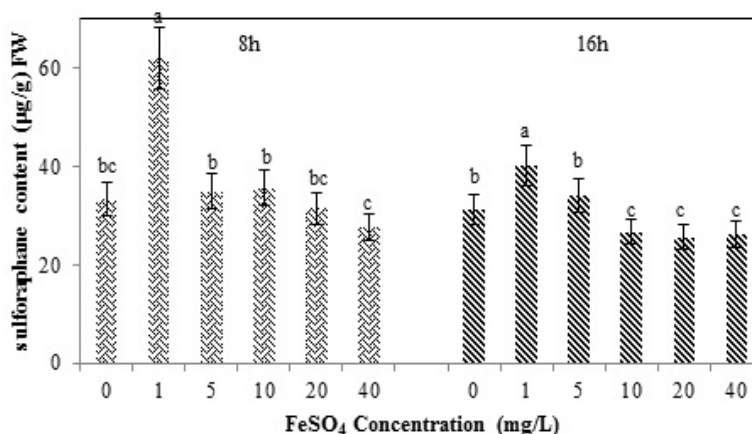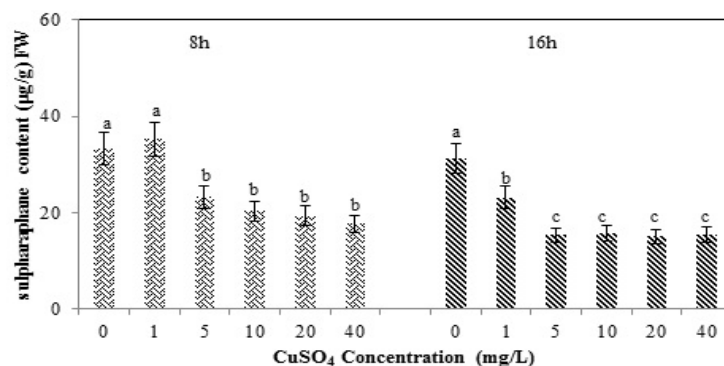**Fig. 1**: HPLC chromatogram of the SFN standard with sample.

Fig. 2: SFN content in *L. draba* seedlings in treatment with different concentrations of Fe ions after 8 and 16 hours. Bars with different letters are significantly different at $p \le 0.05$, according to Duncan's multiple range tests.

The SFN content drastically decreased in the seedlings treated with Cu^{2+} at both treatment times (except for treatment with 1 mg/mL after 8 hours that SFN content was equal to the control) (Fig. 3). The decrease in SFN content was more obvious after 16 hours of treatment in compared with 8 hours.
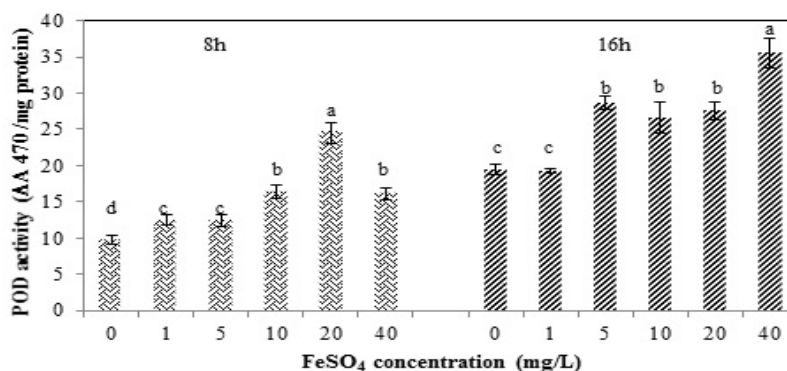
Fig. 3: SFN content in *L. draba* seedlings in treatment with different concentrations of Cu ions after 8 and 16 hours. Bars with different letters are significantly different at p≤0.05, according to Duncan's multiple range tests.

- **Peroxidase activity**

POD activity was gradually elevated with the increase in Fe^{2+} and Cu^{2+} concentration in media after 8 hours of treatment, and reached to the highest amount at 20 mg/L treatments (Fig. 4 and 5) and thereafter decreased. While, treatment for 16 hours, the POD activity increased by the increase metal ions concentration in media, and the maximum activity was seen at the highest concentration for the both metals (Fig. 4 and 5). Furthermore, after 16 hours of treatment more POD activity was detected in compared with 8 hours.

Fig. 4: POD activity in Fe^{2+}- treated *L. draba* seedlings during 8 and 16 hours. Data are presented as the means ± SD (n=3). Bars with different letters are significantly different at p≤0.05, according to Duncan's multiple range tests.
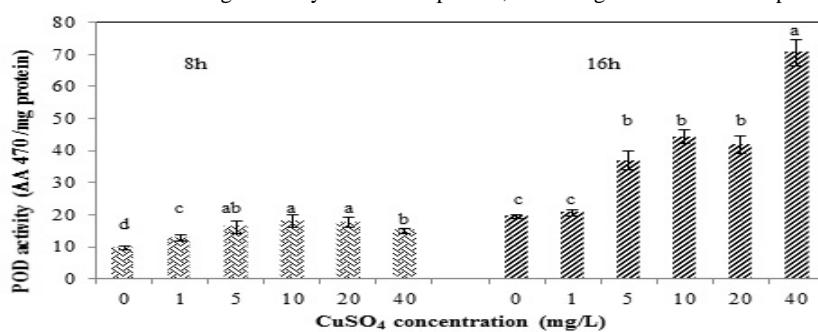
Fig. 5: POD activity in Cu^{2+}- treated *L. draba* seedlings during 8 and 16 hours. Data are presented as the means ± SD (n=3). Bars with different letters are significantly different at p≤0.05, according to Duncan's multiple range tests.

- **Catalase activity**

CAT activity showed no significant changes compared to the control in Fe^{2+}- treated seedlings at the both times of treatment. Although at 1 mg/L concentration of this ion, significant increase in CAT activity was seen after 16 hours treatment. This behavior was similar to the control in Cu^{2+}-treated seedlings after 8 and 16 hours (data not shown).

Discussion

L. draba, a noxious weed of the Brassicaceae family, has been served as a suitable source for glucoraphanin extraction (Powell et al., 2005). SFN,

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an isothiocyanate derived from glucoraphanin, is well known due to its anticancer effects (Lenzi et al., 2014). In this study, SFN production level was analyzed in 7-day-old seedlings of this medicinal plant under treatment with various concentrations of Fe and Cu ions. As previous reports demonstrated that metals can be absorbed more effectively in acidic pH (Taiz et al., 2002), hence, in the experimental conditions, the effects of these elements were analyzed under the acidic condition (pH=5.8).

According to our results, the SFN content drastically increased especially in treated seedlings with the lowest Fe^{2+} concentration after 8 hours treatment. Meanwhile, the increase of Fe^{2+} concentrations in media as well as time of treatment led to reduce of SFN content, considerably after 16 hours treatment. Furthermore, the inhibitory effects of Cu^{2+} on SFN production level were seen at all examined concentrations. This inhibitory effect was more obvious after 16 hours.

In contrast, treatment for 16 hours was accompanied with drastically increase in POD activity in treated seedlings with the both metals (Fe^{2+} and Cu^{2+}).

In plants, POD and CAT are the main enzymes to modulate production level of H_2O_2 via breaking it down into H_2O and O_2 (Bartosz 1997). H_2O_2 considered as a signal of oxidative stress and involved in inactivation of signaling cascades (Mejía-Teniente et al., 2013). It has been shown that the effects of this molecule directly or indirectly through mediated by some plant hormones such as Jasmonate and salicylic acid induce expression of several genes involved in the defense system as well as secondary metabolites biosynthesis pathway (Maksymiec et al., 2005; Maksymiec 2007 & Xu et al., 2007).

In the other hand, positive effects of Jasmonate and salicylic acid on glucosinolates content, have been reported in oilseed rape (*Brassica napus L.*) and cabbage (*Brassica oleracea*) (Kiddle et al., 1994 & Fritz et al., 2010). Glombitza and colleagues (2004) revealed that the expression level of some key genes involved in glucosinolates biosynthesis, changed upon elicitation with the mentioned hormones.

Overall, according to the promoted level of POD activity, it can be concluded that H_2O_2 production was induced along with treatment seedlings with the both metals. Thus, it may be purposed that, in presence of low Fe^{2+} concentration, triggering of H_2O_2 directly or through mediated by plant hormones induce glucoraphanin biosynthesis pathway that in turn resulted in increasing the SFN content.

Uptake and accumulation of several metals (including Fe and Cu ions) by this weed plant has been reported by Chehregani and Malayeri, (2007). Thus, increase in SFN contents in Fe^{2+}-treated seedlings may also be due to the stimulatory effects of this metal on myrosinase activity (Searle et al., 1984 & Uda et al., 1986). Nevertheless, reduction of SFN contents in Cu^{2+}-treated seedlings may be related to the inhibitory effects of Cu ions on myrosinase activity (Liang et al., 2006).

It seems that the high level of H_2O_2 in treated seedlings with higher Fe^{2+} and Cu^{2+} concentrations cause more inducing expression of the genes encoding enzymatic antioxidant system. This is reminiscent with the previously studies that reported expression of some genes encoding antioxidant enzymes (such as CAT) promoted in the treated plants with heavy metals (aluminum) (Rezaee et al., 2013) and exogenous H_2O_2 (Mejía-Teniente et al., 2013).

However, our results showed along with the promotion level of peroxidase activity, no changes were observed in CAT activity. This result is in contrast to several studies that suggested CAT has a key role in elimination of H_2O_2 (Guan et al., 1995; Ghanati et al., 2005 & Mejía-Teniente et al., 2013). Our finding either may be attributed to the high level of POD in *L. draba* as it has been reported in horseradish (a member of the Brassicaceae family) (Soudek et al., 2005) or higher affinity of POD (low k_m) for H_2O_2 rather than CAT.

In general, it may be concluded that Fe^{2+} at low concentrations and short period time of treatment can stimulate many non-enzymatic antioxidant pathways including glucoraphanin biosynthesis more effectively. Conversely, Cu^{2+} and higher concentrations of Fe^{2+} can induce more enzymatic antioxidant pathways. However, further studies are required to analyze all effects of the mentioned ions on glucoraphanin biosynthesis pathway as well as myrosinase activity in this medicinal plant.

Acknowledgments

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