African Journal of Biochemistry Research Vol.3 (4), pp. 077-083, April, 2009
Available online at http://www.academicjournals.org/AJBR
ISSN 1996-0778 © 2007 Academic Journals

Full Length Research Paper

Enhancing antioxidant availability in grains of wheat plants grown under seawater-stress in response to microalgae extracts treatments

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Accepted 28 December, 2008

This study investigated the antioxidant capacity and the levels of enhanced total carotenoids (TCAR), tocopherols (TOC) and phenolic (TPC) and protein (PC) contents in whole grains of wheat plants irrigated 10 and 20% (v/v) seawater (SW) in response to water extracts of microalgae *Spirulina maxima* (SME) and *Chlorella ellipsoida* (CEE) and exogenous plant growth enhancers of ascorbic acid (Vit. C) and benzyladinin (BA) treatments. Significant differences ($P < 0.05$) in amounts of TCAR (ranged 80 to 140 µg/g), TOC (ranged 50.4 to 115 µg/g), TPC (ranged 0.80 to 2.96 mg/g) and PC (ranged 9.34 to 13.79 %) in wheat grains among all treated plants were observed. The levels of their compounds increase related to irrigation-SW combined with algal treatments. The ethanolic extracts of grains of SW-stress plants treated with algal extracts exhibited high antioxidant capacity based on scavenging of DPPH and ABTS radicals than other samples. This activity remarked correlation with levels of antioxidant compounds present in these extracts. The electrophoretic profiles (SDS-PAGE fingerprint) of grains protein of treated samples exhibited similar pattern that in controls samples. It is concluded that the application of algal extracts to wheat plants irrigated SW lead to increase antioxidative components and protein content; hence consumption of these whole grains may render beneficial health effects.

Key words: Microalgae, antioxidant activity, phenolics, proteins, seawater.

INTRODUCTION

Wheat is a major agriculture commodity and dietary component across the world. It is one the most important cereals in view of nutrition values. It serves as rich sources of fiber, vitamins, carbohydrate, proteins and mineral (Fardet et al., 2008). Furthermore, wheat grains contain unique phytochemicals that complement those in fruits and vegetables when consumed together. In general, wheat grains have been known to contain high amounts of various classes of phenolics compound includes phenolic acids, anthocyanidins, quinones, flavonoids and amino phenolic compounds that render potential health benefits (Maillard and Berset, 1995; Shahidi and Naczk, 1995; Lloyd et al., 2000). However, some of these phytochemicals such as ferulic acid and diferulates are generally predominantly found in wheat varieties (about 50 - 67%, of total phenolic acids), but are not present in significant quantities in some fruits and vegetables (Bunzel et al., 2001; Zhou et al., 2004).

Epidemiological studies have shown that regular consumption of whole grains and whole grain products is positively associated with reduced risk of various type of chronic diseases such as cardiovascular disease (Liu, 2007; Anderson et al., 2000), type 2 diabetes (Meyer et al., 2000; Venn and Mann, 2004) some cancers (Kasum et al., 2002) and stroke (Keli et al., 1996). However, wheat grain possesses significant antioxidant activity, which implies the potential for utilizing and promoting the health benefits of wheat (Iqbal and Ashraf, 2006; Fardet et al., 2008). Phenolic compounds found in wheat grains (e.g., phenolic acid, flavoionds and tocopherols) are the main antioxidant compounds that is believed to be responsible for the antioxidant properties (Zhou et al., 2004; Liu 2007 and Fardet et al., 2008). Furthermore, many of these compounds show diverse of bioactivities, such as antimitotic, antiviral, antiallergic, antimutagenicity and antiageing effects (Stavric, 1994; CooK and Samman, 1996; Abd El Baky et al., 2008; Liyana-Pathirana and Shahidi, 2007).

In Egypt, human overpopulation becomes a serious constraint for crop production such as maize, rice and

wheat. Thus, improving overall growth and performance of agriculture crops is an important goal to improve productivity. This is driven by the need to provide food for steadily growing population. Egypt also is present in semiarid region; seawater therefore, has been a resent effort, the possibility of obtaining reasonable yield and quality to products from whole grains (Abd El-Baky et al., 2008). In a previous study involving wheat plants, the cultivated plants were irrigated seawater (10 and 20% levels) and treated with algal extracts enrich in antioxidant compounds such as Vitamin C, carotenoids, tocopheroles and phenolic acids (Abd El-Baky et al., 2008). Due to their inherent antioxidant potential, the wheat plants appear to be enhancing in growth as reflected in both biochemical and agronomic response. Therefore, the present study was carried out, based on hypothesized that the treated of wheat plants with algae extracts may results in an increase of photochemical compounds in grains that possess high antioxidant properties. Thus, the antioxidant contents and antioxidant capacity of different wheat grains was determined. In addition, the protein content and protein profile was investigated in order to determine the bread-making quality of their wheat grains.

MATERIALS AND METHODS

Samples

The different samples were obtained from harvest wheat crops after cultivation under different treatments. Controls samples of the wheat grains (*Triticum aestivum* L., cv. Giza 94) were obtained from plants irrigated either 10 and 20% seawater (SW-stress, v/v) or natural water (NW non-stress) though with different growth stages (GS). Treated samples were obtained from wheat plants irrigated 10 and 20% seawater and treated intervals at 40 and 70 d of GS, with either microalgae water extracts of *Chlorella ellipsoida* and *Spirulina maxima* (at concentration of 5g /L in 0.1% tween 20) or standard plant growth enhancers (ascorbic acid and benzyladinine, at concentration of 200 mg/L). The data of biochemical parameters of treated samples were compared with those data of either 10 or 20% SW-stress and NW non-stress plants.

Extraction of crude phenolics of wheat

The crude phenolic compounds of whole grains of different samples were extracted with 80% aqueous ethanol (1:10, w/v) at 4°C for 16 h. The resulting slurries were centrifuged at 6000 xg for 5 min and the supernatants were saved. The residues were re-extracted (1:5 w/v; 80% ethanol) under the same conditions. The supernatant of both extractions were combined and was concentrated under vacuum at 40°C to give a semisolid. Yields of the crude phenolic extracts were reported as percentage of defatted materials (Liyana-Pathirana and Shahidi, 2007). All samples were protected permanently from light.

Determination of total phenolics content (TPC)

Total phenolic contents of crude ethanolic extracts were determined following the method by Singleton et al. (1999). A 100 µl aliquot of sample (2 mg of extract/ 1 ml of ethanol) was added to 1 mL of 10% Folin–Ciocalteu phenol reagent. After 4 min incubation at room temperature, 2 mL of 5% sodium carbonate solution was added. The reaction mixture was incubated for 45 min in a dark-cupboard, the absorbance at 725 nm was then determined. Ferulic acid (10 - 100 µg/mL of 95% ethanol) was used as phenolic standard for preparation of calibration curve. Phenolic contents are expressed as micrograms of ferulic acid equivalents (FAE) per gram.

Determination of tocopherols by HPLC

Tocopherols were analyzed by HPLC method. HPLC system equipped with Spectra system UV2000 detector at 290 nm and separated on Vydac analytical column (25 cm X 4.6 mm i.d., 5 µm particle sizes). Tocopherols were eluted with acetonitrile: methanol (9:1 v/v) at a flow rate of 1 mL min^{-1}. Standard of α-tocopherol was run under the same conditions (Abd El-Baky et al., 2003).

Determination of total carotenoids

The total carotenoids in wheat grains samples were spectophotometrically determined at 450 nm according to AOAC standard methods (1995). A standard curve was established using β-carotene in hexane.

Antioxidant assays

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The method described by Tagashira and Ohtake (1998) was used in order to assess the DPPH radical scavenging of ethanolic wheat extracts. A 0.1 mM (final concentration) DPPH in ethanol solution was mixed with wheat extracts and vortexed thoroughly. The absorbance of the mixture at ambient temperature was measured at 519 nm, for 60 min at 10 min intervals. The scavenging of DPPH was calculated according to the following equation: scavenging percenttage (%) = [(A_{517} control - A_{517} sample) / A_{517} control] X 100; where: A control = absorbance of DPPH radical + methanol; A sample = absorbance of DPPH radical + wheat extract/ standard. The scavenging capacity was expressed as l mol DPPH radical scavenged/g of defatted material.

Measurement of total antioxidant activity

Total antioxidant activity was determined according to the Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay described by Re et al. (1999). The extracts and reagents were prepared in a 0.1 M phosphate buffer saline (PBS, pH 7.4). A $ABTS^{\cdot+}$ solution (2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) was prepared by mixing 2.5 mM potassium persulfate with 7.0 mM $ABTS^{\cdot+}$ in a 1:1 (v/v) ratio, and leaving the mixture for 8 h. The absorbance of the radical solution at 743 nm was 0.7 ± 0.03. The solution was stored at room temperature and protected from light. Trolox standard curve (ranged 0.5 - 20 µg/mL) was prepared. The reduction in the absorbance of the $ABTS^{-2}$ solution (1.9 mL) at different concentrations of Trolox (2 - 30 µg/mL) over a period of 6 min was measured and plotted. The TEAC values of wheat extracts (5 mg/mL) were determined in the same way and expressed as µM Trolox equivalents.

Determination of total protein content

A known weight of fine powdered seeds (Ca, 0.2 g) was digested using a micro Kjeldahl apparatus. The total protein content was calculated by multiplying the total nitrogen by 5.75 (AOAC, 1995).

Table 1. Changes in antioxidant compounds and total protein contents of wheat grains irrigated seawater in response to algal extracts and plant growth enhancer's treatments.

Treatments	µg /g D.W	Ratio[a]	µg/ g D.W	Ratio[a]	mg /g D.W	Ratio[a]	% D.W
Irrigation natural water (NW) only	80.39		50.21		0.80		11.25
NW+*S. maxima*	125.37	1.56	98.12	1.95	1.43	1.80	13.79
NW +*C. ellipsoida*	110.35	1.37	91.25	1.82	1.33	1.70	13.23
NW +vit C	90.11	1.12	72.11	1.44	0.82	1.03	11.45
NW +BA	85.17	1.06	65.14	1.30	0.95	1.20	11.93
Irrigation 10% sea water (SW) only	84.78	1.05	55.14	1.1	1.02	1.30	9.95
SW+*S. maxima*	134.10	1.67	110.25	202	2.31	2.90	13.21
SW +*C. ellipsoida*	117.38	1.4	98.14	1.95	1.72	2.15	13.12
SW +vit C	95.17	1.18	79.35	1.58	1.21	1.52	11.96
SW +BA	89.15	1.11	68.27	1.35	1.43	1.80	10.29
20% sea water only	90.84	1.13	58.36	1.16	1.53	1.92	9.34
SW+*S. maxima*	140.98	1.75	115.14	2.30	2.96	3.71	13.23
SW +*C.ellipsoida*	124.32	1.55	105.24	2.10	2.14	2.70	13.21
SW +vit C	98.35	1.22	82.21	1.60	1.45	1.81	11.11
SW +BA	92.36	1.15	73.25	1.45	1.92	2.40	10.53
LSD at level (P < 0.01)	2.50		1.64		0.12		0.95

Ratio[a]: Treatment / Negative control (irrigated only by natural water)
All values are significant at (P ≤ 0.05), ± S.D
Data present the mean of three experiments with replicated measurements.

Separation of grains protein by SDS-PAGE

Protein extracted by grinding 0.5 g of wheat grains mortar in liquid nitrogen and 5 ml buffer solution (containing 250 mM sucrose and 25 mM Tris, pH 7.2). SDS-PAGE was performed based on the method described previously by Laemmli (1970).

Statistical analysis

All analyses were performed in triplicate and data reported as mean ± standard deviation, unless otherwise stated. Analysis of variance was done using the COSTAT computer package (Cohort Software, CA, USA). The mean values were compared with LSD test.

RESULTS AND DISCUSSION

The amounts of total carotenoids (TCAR), tocopherols (TOC) and phenolic content (TPC) of whole-wheat grains of plants irrigated either 10 and 20% seawater (SW-stress plants) or irrigated natural water (NW non-stressed) in response to algal water extracts, Vitamin C and BA treatments, are shows in Table 1. The concentration of these components in whole-wheat grains showed a significant difference (P < 0.05) among all treatments. Irrigation of wheat plants with 10 and 20% SW caused significant increase in accumulation of TCAR, TOC and TPC in yielded grains. The levels of those compounds being about 1.05, 1.1 and 1.3 and 1.13, 1.16 and 1.92 times as high as that in grains of plants irrigated NW, respectively. Thus, irrigation with SW increased concentration low molecular mass antioxidant compounds in yield wheat crops. Application of extracts of blue green algae, *S. maxima* and green algae, *C. ellipsoida* on wheat plants irrigated SW led to accumulation of high amounts of antioxidant compounds in yielded grains as compared to that in plants irrigated 10 and 20% SW. The highest levels of TCAR, TOC and TPC in wheat grains were found in plants irrigated 20% SW and treated with extracts of *S. maxima* (124.32, 105.24 and 2.1 mg/g of defatted wheat grains (DWG), respectively), followed by *C. ellipsoida* (124.32, 105.24 and 2.1 mg/g DWG). Similar trained was observed in grains of plants irrigated 10% SW and treated with *S. maxima* (134.1, 110.25 and 2.3 mg/g, respectively) and *C. ellipsoida* (117.38, 98.14 and 1.72 mg/g, respectively). On the other hand, the concentration of TCAR, TOC and TPC in yielded grains of wheat plants irrigated either 10 or 20% SW were affected by treated with Vitamin C and BA when compared to plants irrigated NW values, and there was no effect as compared to those values found in plants irrigated SW only. Thus, the concentration of antioxidant compounds was significantly

Table 2. DPPH Scavenging Capacity % of wheat grains extracts of plants irrigated sea water combined with algal extract treatments.

Treatments	IC_{50}^{b} (μg mL^{-1})	DPPH Scavenging Capacity %
BHA	13.8	92.00
BHT	15.6	94.00
α-Tocopherol	19.0	82.00
Irrigation natural water (NW) only	105.5	13.25
NW *+S. maxima*	30.8	55.21
NW *+C.ellipsoida*	35.2	42.31
NW + vit. C	40.9	29.32
NW +BA	50.8	23.52
Irrigation 10% sea water (SW) only	90.5	16.24
SW *+S. maxima*	25.4	59.21
SW *+C.ellipsoida*	32.4	46.21
SW +vit. C	38.6	35.27
SW +BA	42.1	28.31
20% sea water (SW) only	85.9	19.21
SW *+ S. maxima*	24.2	62.12
SW *+C. ellipsoida*	33.5	48.21
SW + vit. C	37.3	37.85
SW +BA	39.6	31.24
LSD at level (P< 0.01)	1.45	

IC_{50}^{b}: Concentration (μg/ml) for a 50% inhibition was calculated from the plot of inhibition (%) against wheat grains extracts concentration
Tests were carried out in triplicate

increased in yielded grains of wheat plants irrigated SW in response to application of algal extracts, when compared with all controls plants include: plants irrigated only NW and either 10 and 20% SW. Furthermore, application of algal extracts had a significant greater effect than standard growth enhancers (SGE, with Vitamin C or BA) in increasing antioxidant contents. However, the order of antioxidant compounds content in yielded grains of wheat plants irrigated SW in response to application with algae extracts and SGE was *S. maxima* > *C. ellipoida* > Vitamin C > BA. This finding clearly suggests that antioxidant compounds in grain of plants irrigated SW were significantly influenced in response to *S. maxima* and *C. ellipsoida* algae extract treatments. In previous studies with wheat, an increase in concentration of low molecular mass antioxidant compounds includes GSH, carotenoids, tecopherols and phenols compounds in wheat leaves of plants irrigated seawater up to 20% correlated with treated with microalgale extracts enrich in antioxidant constituents (Abd El-Baky et al., 2008). Generally, this study showed that the levels of phenolic, carotenoids and tocopherols content in Giza-94 wheat grains were comparable to previous findings in several varieties of wheat located in Asia and North America (Iqbal and Ashraf, 2006). However, it is know that grain types, varieties, agronomic, the part of the grain sampled and environmental factors had significant affect on the concentrations of antioxidant compounds in whole grains (Adom et al., 2005).

Radical scavenging capacity

Free radical scavenging ability of various wheat grains ethanolic extracts of SW-stressed plants in response of treatments was measured with the change of absorbance caused by the reduction of DPPH radical, and results of percentage scavenging activity (% SC) and the values of IC_{50} are shown in Table 2. High %SC was an indication of high degree of scavenging DPPH radical and high antioxidant activity. It was clear that the grains ethanolic extracts showed significant scavenging activities with various degrees (IC_{50} values ranged from 24.2 to 105.5 μg/mL). The highest scavenging activity (P < 0.5%) was found for grains extracts of plants irrigated 10 and 20% SW combined with treated by extracts of *S. maxima* (% SC, 59.21 and 62.12%), followed by *C. ellipsoida* (% SC, 46.21 and 48.21%) and then that treated with Vitamin C (% SC, 35.27 and 37.85%) and BA (% SC, 28.31 and 31.24%). Thus, scavenging activities of extracts of SW-stressed plants treated with algal extracts were apparently higher than those treated with BA and Vitamin C. However, scavenging activity from wheat grains of stressed plants treated by algal extracts (% SC, 46.21 – 62.12%) was lower compared with that of commercial antioxidant BHT (94%) and BHA (92%).

$ABTS^{\cdot+}$ scavenging activity: As shows in Table 3, the ethanolic extracts of wheat grains of plants irrigated SW

Table 3. $ABTS^{\cdot+}$ Scavenging capacity % of wheat grains extracts of plants irrigated sea water combined with algal extract treatments.

Treatments	$ABTS^{+}$ scavenging activity
	µmol trolox /g grain
Irrigation natural water (NW) only	8.74
NW+ *S. maxima*	17.36
NW + *C. ellipsoida*	15.66
NW +vit. C	12.35
NW +BA	10.36
Irrigation 10% sea water (SW) only	10.25
SW+ *S. maxima*	18.24
SW + *C. ellipsoida*	16.94
SW +vit. C	13.65
SW +BA	11.57
Irrigation 20% sea water (SW) only	12.54
SW+ *S. maxima*	20.14
SW + *C. ellipsoida*	18.35
SW +vit. C	15.14
SW +BA	13.57
LSD at level (P< 0.01)	1.21

combined with treated by algal extracts and PGEs exhibited appreciable $ABTS^{\cdot+}$ scavenging activity. These activities were varied significantly from one anther. The $ABTS^{\cdot+}$ scavenging activity was in the range of 20.14 and 8.74 µM of trolox / g grains. The highest $ABTS^{\cdot+}$ scavenging activity were observed for extracts of wheat grains of SW-stressed plants treated with *S. maxima* (ranged 17.36 to 20.14 µmol of trolox/ g grains), followed by *C. ellipsoida* (ranged 15.66 to 18.35 µmol of trolox/ g grains) and then Vitamin C (ranged 12.35 to 15.14 µmol of trolox/ g grains) and BA (ranged 10.36 to 13.57 µmol of trolox/ g grains). In general, the ability to scavenge both DPPH and ABTS radicals by ethanolic extracts of grains of SW-stressed wheat plants in response to algae extracts and reference PGEs treatments was in the order of *S. maxima* > *C. ellipsoida* > Vitamin C > BA.

This finding was in accordance with that study on alcoholic extracts of a wheat variety grown in different location (Canadian and Pakistanis), which showed good antioxidant activity with various degrees (Iqbal and Ashraf, 2006). Moreover, in a series of *in vitro* antioxidant assays, the alcoholic extracts of some wheat varieties (whole-grain) and their constituent fractions (such as wheat germ, bran and flour) exhibited significant antioxidant properties against many free radicals scavenging models (Zhou et al., 2005; Fardet et al., 2008). Iqbal and Ashraf (2006) and Liyana-Pathirana and Shahidi (2007) reported that the wheat ethanolic extracts contains large amounts of phenolic compounds possessed high antioxidant activity due to their hydrogen-donating ability. Therefore, it could be possess hydroxyl radical scavenging properties in both non-enzymatic lipid peroxidation and deoxyribose assays. Again, the present results suggested that alcoholic extracts of wheat grains contains high amounts of antioxidative components included TCAR, TOC and TPC (Table 2). Their compounds having a potent antioxidant activity (Adom, et al., 2003; Zhou et al., 2005; Abd El Baky et al., 2007). Recently, positive correlation between free radical scavenging activity of wheat grains and their fraction and phenolic contents was reported (Zhou et al., 2005; Choi, et al., 2007; Liu, 2007). However, in this study, ethanolic extracts of wheat grains of SW-stressed plant treated with growth enhancer (Vitamin C and BA) possess a week antioxidant activity than that treated with algal extracts. Although, their extracts contains a significant amounts of phenolic compounds compared to those of the others extracts. It is though that other components in wheat ethanolic extracts, such as carotenoids and tocopherols, may be affected in the antioxidant activity.

Consequently, antioxidant activity may vary depending on the actual composition of these extracts. Finally, these findings suggest that wheat grains of SW-stressed wheat plants exhibited a marked antioxidant potential and consequently, can be uses as a rich sources of antioxidant compounds, which play a significant role in protective our bodies against oxidative radicals, which cause various types of chronic diseases.

Protein contents and protein profile

As show in Table 1, the amount of total proteins content (TPs) varied significantly among whole wheat grains of non-stressed and SW-stressed plants treated with either

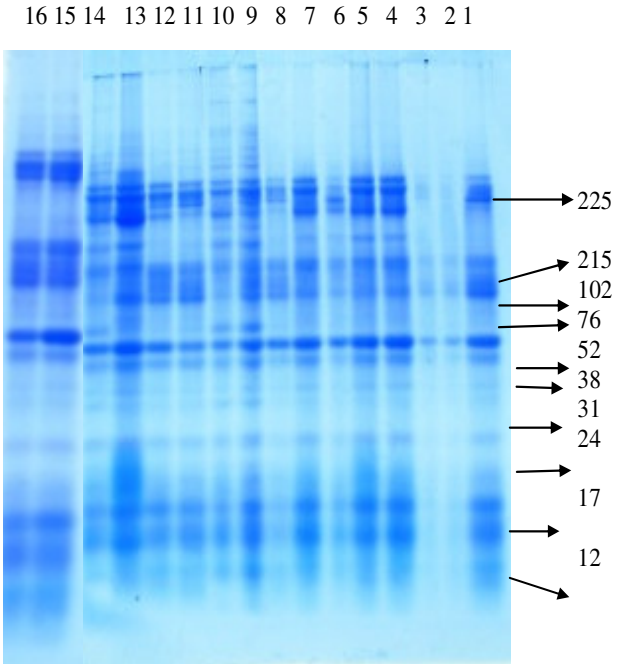

Figure 1. SDS-PAGE pattern of wheat grains as affected by algal antioxidant spraying and irrigated with sea water.

1. Protein marker; 2. Natural water only; 3. Sp. antioxidant; 4. Chl. antioxidant; 5. Vit. C. 6. BA; 7. 10% sea water only; 8. Sp. antioxidant; 9. Chl. antioxidant; 10. Vit. C; 11. BA. 12. 20% sea water only; 13. Sp. Antioxidant; 14. Chl. antioxidant; 15. Vit. C; 16. BA

algae extracts or PGEs. The differences were also significantly ($p < 0.5$) between algal extracts and PGE treatments. However, the grains of plants irrigated 10 and 20% SW combined with application by algal extracts of *S. maxima* (13.21 and 13.23%) and *C. ellipsoida* (13.12 and 11.21%) had higher TPs contents compared with both that in plants irrigated 10 and 20% SW only (9.95 and 9.34%), and that in SW-stressed plant treated by PGEs (either with Vitamin C: 11.96 and 11.11% or BA, 10.29 and 9.53%). Thus, the ability to increase protein content in wheat grains of SW-stressed plants was in the order of *S. maxima* > *C. ellipsoida* > Vitamin C > BA. Furthermore, the grains of non-stressed plant treated with algal extracts or PGE had higher levels of protein content than that in grains of Sw-stress plants in response to the same treatments, but was not significantly (Table 1). Therefore, these results suggest that the treatment of wheat plants with algal extracts and PGE have significant effect on stimulation of protein synthesis. This finding is compatible with that found by Abd El Baky et al. (2008), which is the application of algal extracts on SW-stressed plants during the growth stages leading to increased of endogenous protein synthesis.

SDS-Polyacrylamide gel electrophoresis

The electrophoretic profiles (SDS-PAGE fingerprint) of grains protein of SW-stress wheat plants treated either with algal extracts or PGE compared to those of the (controls) non-stressed, 10 and 20% SW- stressed plants are shown in Figure 1. As expected the treated of SW-stress plants had an approximate similar protein profile [among all protein groups, high (> 80 kDa, HMW), medium (<80 - >40 kDa, MMW) and low molecular weight (<40 kDa, LMW)] that was found in all controls plants (Figure 1, lanes 2 - 16), This finding indicated that no recognizable losses of bands in protein grains of stressed plants due to application of algal extracts through growth stages. However, there were some new protein bands (Figure 1, lanes 12 - 14) corresponding to molecular of approximate weights from 225 to 215 kDa (lanes 13 and 14). In addition, the strong relative intensity of some bands in LMW proteins was present in the wheat grains of 20% SW-stressed plants treated with algal extracts. This phenomena indicating that the new HMW proteins were induced in grains of stressed plants in response to algal application. According to Huang and Khan (1997) HMW glutenin subunits are the most important components in determining wheat grains quality. That HMW and LMW proteins may be randomly linked leading to increase strength of the gluten polymer network. Therefore, the wheat grains contain high amounts of HMW glutenin had good bread-making quality (Payne et al., 1979). Finally, it could be concluded that the application of algal extracts to wheat plants irrigated SW showed a positive effects on antioxidative components and protein contents; hence consumption of these whole grains may render beneficial health effects.

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