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Levels of antioxidant enzymes and alkaline protease from pulp and peel of sunflower

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ABSTRACT

Objective: The activity of enzymes participating in the systems of antioxidant protection was assayed in the peel and pulp of sunflower. The essential roles of proteases in food stimulate research to find other sources of the enzyme especially from non-conventional sources. In the present work, we study several biochemical parameters in the pulp and peel of sunflower.

Methods: Pulp and peel of sunflower was extracted, antioxidant enzymes and non-enzymatic antioxidant were measured. Alkaline protease was measured and purified from pulp in sunflower.

Results: High carbohydrate concentration, beta-carotene, catalase and ascorbate peroxidase activities, free radical scavenging capacity and free flavonoid content were observed in the peel of sunflower. Whereas, MDA and ceruloplasmin activities were high in the pulp of sunflower.

Conclusions: The present study concluded that peel in sunflower are strong radical scavengers and can be considered as good sources of natural antioxidants for medicinal and commercial uses. Further analysis showed that protease activity was a significantly high in the pulp compared to the peel.

1. Introduction

Sunflower (*Helianthus annuus* L.) is important for its oil. It is grown under dry land conditions and, several studies reported that substantial yield performance decreases under water stressed conditions [1]. It is grown mainly in many countries in the world especially in Russia, Argentina, China and France, which are the highest global producers [2].

Different condition causes several variations in plant metabolism like osmotic stress and formation of reactive oxygen species (ROS). The ROS production is removed by an antioxidant compounds and antioxidant enzymes like ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) [3,4]. The ROS contribute mainly in the initiation and development of lipid peroxidation through induce oxidative stress [5]. In a cell enzyme SOD in the first line of defense against reactive oxygen species [6]. The SOD detoxifies superoxide anion to hydrogen peroxide and molecular oxygen by catalyzing the dismutation, which acts as a metalloprotein to work in the reactions in the mitochondria, cytosol and chloroplasts [6]. The 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical which has an unpaired valence electron at one atom of nitrogen [7]. The DPPH has been widely used for antioxidant capacity screening and estimation due to its clear reaction mechanism, solvent compatibility and the technical simplicity of its assays which requires no special equipment [7]. Phenolics are compounds possessing aromatic rings with hydroxyl groups. When are in diet, these compounds provide health benefits associated with reduced risk of chronic disease [8].

Enzyme hydrolysis is commonly using for development of functional properties of food proteins [9]. Proteases are protein-digesting enzymes which are categorized depending on the

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optimal operating pH; neutral, alkaline and acidic proteases, it can be found in all living creatures [10]. Proteases play a main role in biotechnology and are generally used in the tanning industry, in bioremediation processes, in the manufacturing of biological detergents, pharmaceutical industry and peptide synthesis [11].

The objective of current study is to investigate several antioxidant parameters in peel and pulp of sunflower, as well as to extract, purify and characterize of alkaline protease of pulp sunflower.

2. Materials and methods

One gram sample was homogenized with 5 mL coldly extraction buffer (0.1 M potassium phosphate buffer, pH 7.8). The homogenate was separated by centrifugation at 4 °C for 30 min. Supernatant was used as a crude extract for measurement of several biochemical parameters.

The activity of catalase was measured according Pereira *et al.* method [12]. The H_2O_2 concentration was determined according to Rummun *et al.* [13]. Measurement of ascorbate peroxidase activity (APX) was conducted according to Nakano and Asada method [14]. The activity of ceruloplasmin oxidase was measured according the modified method of Rice [15]. Malondialdehyde (MDA) content was determined by Bailly *et al.* [16]. Beta carotene concentration was measured according to Santra method [17]. Estimation of total phenols content (TPC) using folin is based on the reaction between phenol and oxidizing agent phosphomolybdate [18].

The free radical scavenging capacity via 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was used to determine the antioxidant activity [8]. Flavonoid contents in the extract were determined using colorimetric method [19]. SOD activity [U/mg (protein)] was determined using the spectrophotometric method [20].

The reducing sugar was measured according to McDonald and Chen [21]. Measurement of fatty acid was determined according to Velikova *et al.* [17]. The protease activity was measured according to McDonald and Chen [21]. Protein concentration was determined by Lowery method [22].

To purity alkaline protease from the crude extract, 50% saturation of ammonium sulphate were utilized and placed in ice bath at 0 °C and kept at 4 °C for overnight. The resulting suspension after ammonium sulphate precipitation was centrifuged at 10 000 *g* for 30 min at 4 °C, followed by dialysis in dialysis sac overnight at 4 °C using sodium bicarbonate. Gel filtration chromatography was used to partially purify protease. The column was packed with Sephadex G-100 (120X2) cm in a glass column and equilibrated with 0.1 M Tris–HCl buffers (pH 10) [23]. Enzyme activity and protein of purified alkaline protease were measured.

To characterize the enzyme, several independent experiments were carried out as the following. Alkaline protease reaction was conducted at optimum condition using different concentration of casein as a substrate [0.50, 0.10, 0.15, 0.25, 0.40, 0.50, 0.60, and 0.70] mg/mL. The data obtained were used to plot Lineweaver–Burk graph to calculate Km and Vmax values for alkaline protease.

The effect of temperature on enzyme was conducted by carrying out the enzyme reaction at different temperatures [25, 30, 35, 40, 45, 50, 55, 60, and 65]. The pH optimum was assessed by different pH [7,8,9,9.5,10,11,12] for alkaline protease. The activation energy (Ea.), also free energy (ΔG*), enthalpy (ΔH*), and entropy (ΔS*) of the transition state were determined. The thermodynamic factors of the transition state were calculated from Arrhenius plot.

Statistical analysis of data was performed by SPSS [version 21.0]. The significance difference between mean values was carried out by student T-Test.

3. Results

The results showed higher concentration of fatty acid, carbohydrate, H_2O_2, β-carotene, protein, TPC, MDA and activity of APX, CAT, free radical scavenging capacity and free flavonoid content in peel, while ceruloplasmin, and protease activity were lower in peel when compared with pulp (Table 1). The result showed no significant different in SOD activity (Table 1). The result exhibited a significant low level of alkaline protease activity in peel compared with the pulp (Table 1).

The result showed one peak by gel filtration separations (Figure 1). The specific activity of the enzyme was increased in 4.73 folds than the activity in initial extract (Table 2) for sample.

The maximum activity of the enzyme was obtained by using [0.40 mM] of casein (Figure 2). A linear relationship was shown before reaching Vmax at 31.45 U/mL and Km value of [0.142 mg/mL].

Table 1
The mean of biochemical parameters in pulp and peel in sunflower.

Parameters	Pulp (Mean ± SD)	Peel (Mean ± SD)
Fatty acid [mg/100 gm]	28.59 ± 3.15	31.48 ± 1.85
Carbohydrate [gm./100 mL]	2.57 ± 0.45*	3.47 ± 0.37
H_2O_2 [μmol./gm. FW]	3.12 ± 0.46	3.28 ± 0.52
β-carotene [ppm]	6.98 ± 1.05*	8.22 ± 1.13
Protein [mg/mL]	1.57 ± 0.15	1.74 ± 0.25
TPC [μgm/gm]	2.19 ± 0.43*	4.68 ± 0.31
MDA [mmol/gm]	0.030 ± 0.003*	0.012 ± 0.0001
Ceruloplasmin Oxidase [mg/dL]	9.42 ± 1.11*	6.98 ± 0.79
CAT [U/mL]	245.2 ± 22.25**	216.9 ± 29.5
APX [U/mL]	9.56 ± 1.05**	14.89 ± 1.32
Free radical scavenging capacity [%]	63.14 ± 1.05*	69.30 ± 1.23
Free flavonoid content [μg/g]	555.33 ± 28.22*	569.41 ± 20.05
SOD [U/mg]	4.86 ± 1.08	4.52 ± 1.00
Protease [U/mL]	29.31 ± 0.35***	4.25 ± 0.10

*$P \leq 0.05$; **$P \leq 0.01$ and ***$P \leq 0.001$.

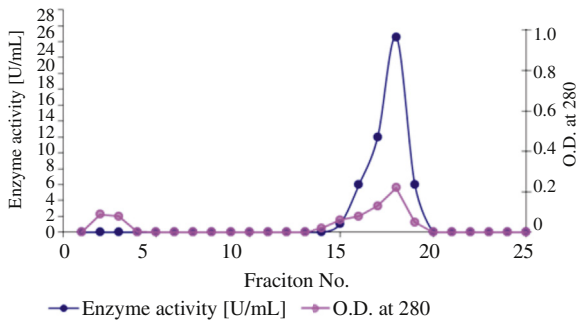

Figure 1. Atypical elution profile for the chromatography alkaline protease.

Table 2
Purification summary of an alkaline protease from pulp in sunflower.

Purification Stage	Volume [ml]	Activity U/mL	Total Activity [U]	Total Protein [mg]	S.A [U/mg] protein	Yield	Folds of Purification
Enzyme Crude	12	31.10	373.2	21.9	17.04	100	1
Ammonium Sulfate Precipitation	10	22.5	225.00	11.55	19.48	60.29	1.14
Dialysis	5	18.50	92.50	3.30	28.03	24.79	1.64
Sephadex G-100	2	24.6	49.2	0.61	80.66	13.18	4.73

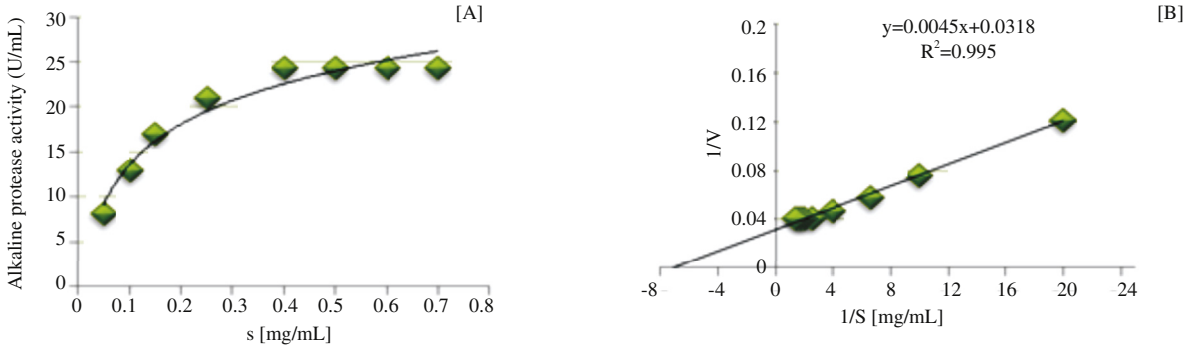

Figure 2. Determination of Km (A) and Vmax (B) in partial purified protease.

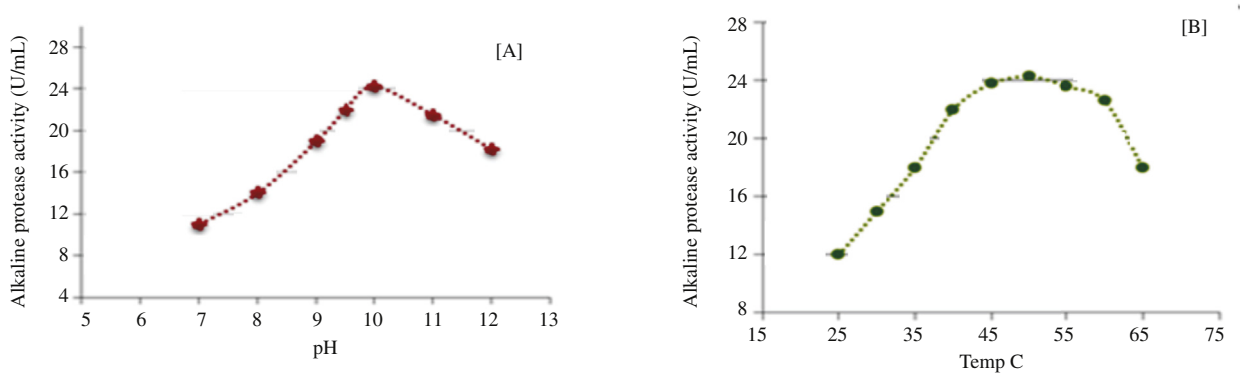

Figure 3. Effect of pH (A) and temperatures (B) in alkaline protease activity.

The current result indicated that maximum enzyme activity was at pH 10 (Figure 3A), and the maximum incubation temperature at 50 °C (Figure 3B).

Arrhenius plot was used to calculate thermodynamic factors of the transition by plotting ln K+1 values against (1/T) values (Figure 4). A linear relationship was obtained with the activation energy of 16.29 kJ/mol and also free energy (ΔG*) was 95.61 kJ/mol, enthalpy (ΔH*) was 13.60 kJ/mol, and entropy (ΔS*) was 253.90 J/mol K of the transition state were determined.

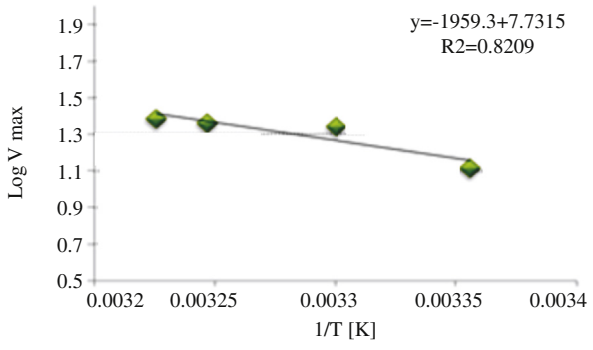

Figure 4. Arrhenius plot.

4. Discussion

In many countries, production of sunflower seeds is for the development of oil and also for their use as toppings in snacks. Sunflower seeds are excellent sources of energy and are often utilized for livestock feed sources of energy and are often utilized for livestock feed [24]. Plants have two methods for detoxifying the H_2O_2 produced [25]. CAT removes ROS produced in different stress conditions and avoid oxidant damage [26]. In seed germination, the increase of MDA contents in endosperms and cotyledons suggest that lipid peroxidation increases through germination process [27].

The antioxidant activity of flavonoids results from the combination of their iron chelating activity and their ability to scavenge ageing-inducing free radicals. Flavonoids can inhibit oxidases such as cyclooxygenase, lipooxygenase, NADPH oxidase and xanthine oxidase, thus preventing the in vivo formation

of ROS and organic hydroperoxide. Furthermore, it has been found that flavonoids stimulate enzymes with well-known antioxidant properties, such as CAT and SOD [28]. The present study shows peel in sunflower are strong radical scavengers and can be considered as good sources of natural antioxidants.

An enzyme with low Km has more affinity for its substrate. Previous studies showed that maximum activity was achieved when casein was used as a substrate [29,30].

The velocity of enzyme-catalyzed reactions depends on pH. Enzymes have pH optimum and frequently give bell-shaped curves of velocity against pH, even though other shapes. Our results agreed with other studies of different sources [31,32]. Temperature is a main factor for maximum activity of enzyme as well as for numerous industrial applications; relatively high thermo stability is desirable for characteristic of an enzyme [33]. Previous study showed a maximum activity at 40 °C while, further increase in temperature leads to decrease the activity and showing 80% loss in activity at 70 °C [30], while another study described maximum enzyme activity of alkaline protease from Aspergillus niger at 45 °C [34].

Characterization and environmental friendly potential application of alkaline protease from pulp in sunflower (*H. annuus*) were studied. The purified enzyme showed maximum activity of 31.45 U/mL with its corresponding Km value of 0.142 mg/mL. The specific activity and substrate affinity of alkaline protease from pulp in sunflower is greater than those of other reported; therefore, it is concluded that it may be potentially useful for industrial purposes. We concluded that peel in sunflower are strong radical scavengers, also it might be sources of natural antioxidant for medicinal and commercial uses.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Inoka K, Dahanayake N. Effect of plant growth regulators on micropropagation of Sunflower (Helianthus annuus L). *Int J Sci Res Publ* 2015; **5**(1): 01-5.

[2] Ribeiro S, Nicacio A, Zanqui A. Application of enzymes in sunflower oil extraction: antioxidant capacity and lipophilic bioactive composition. *J Braz Chem Soc* 2016; **27**(5): 834-40.

[3] Rao A, Ahmad S, Sabir S, Awan S, Shah AH, Khan MF, et al. Antioxidant activity and lipid peroxidation of selected wheat cultivars under salt stress. *J Med Plants Res* 2013; **7**(4): 155-64.

[4] Taghvaei M, Jafari SM. Application and stability of natural antioxidants in edible oils in order to substitute synthetic additives. *J Food Sci Technol* 2015; **52**(3): 1272-82.

[5] Thanh T, Thanh H, Thi Minh HP, Thi Thu HL, Duc LV. Protective effect of Tetracera scandens L. leaf extract against CCl4-induced acute liver injury in rats. *Asian Pac J Trop Biomed* 2015; **5**(3): 221-7.

[6] Yan H, Mao P, Sun Y, Li Li M. Impacts of ascorbic acid on germination, antioxidant enzymes and Ultrastructure of embryo cells of aged elymus sibiricus seeds with different moisture contents. *Int J Agric Biol* 2016; **18**: 176-83.

[7] Islam R, Islam A, Mazumder K. In vitro antioxidant activity of methanolic extract of Helianthus annuus seeds. *J Med Plants Stud* 2016; **4**(2): 15-7.

[8] Nadeem M, Anjum F, Hussain S, Khan M, Shabbir M. Assessment of the antioxidant activity and total phenolic contents of sunflower hybrids. *Pak J Food Sci* 2011; **21**(1–4): 7-12.

[9] Nosenko T, Zhukova Y, Cherstva A. Sunflower protein hydrolysis degree by proteases. *Sci Work Univ Food Technol* 2015; **LXII**: 64-7.

[10] Shine K, Kanimozhi K, Panneerselvam A, Muthukumar C, Thajuddin N. Production and optimization of alkaline protease by Bacillus cereus RS3 isolated from desert soil. *Int J Adv Res Biol Sci* 2016; **3**(7): 193-202.

[11] Al-Askar AA, Rashad YM, Hafez EE, Abdulkhair WM, Baka ZA, Ghoneem KM. Characterization of alkaline protease produced by Streptomyces griseorubens E44G and its possibility for controlling Rhizoctonia root rot disease of corn. *Biotechnol Biotechnol Equip* 2015; **29**(3): 457-62.

[12] Pereira G, Molina S, Lea P. Activity of antioxidant enzyme in response to cadmium in Crotalaria juncea. *Plant Soil* 2002; **239**(1): 123-32.

[13] Rummun N, Somanah J, Ramsaha S, Bahorun T, Neergheen-Bhujun VS. Bioactivity of nonedible parts of Punica granatum L.: a potential source of functional ingredients. *Int J Food Sci* 2013: 602312.

[14] Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate- specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 1981; **22**: 867-80.

[15] Feraye G, Aysen A. Serum ceruloplasmin levels in ewes fed deficient -energy during late pregnancy. *J Animal Veterinary Adv* 2010; **9**(4): 820-5.

[16] Bailly C, Benamar A, Corbineau F, Dome D. Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seed as related to deterioration during accelerated aging. *Physiol Plant* 1996; **97**: 104-10.

[17] Badakhshan H, Moradi N, Mohammadzadeh H, Zakeri MR. Genetic variability analysis of grains Fe, Zn and beta-carotene concentration of prevalent wheat varieties in Iran. *Intl J Agri Crop Sci* 2013; **6**(2): 57-62.

[18] Velikova V, Yordanov I, Edreva A. Oxidative stress and some antioxidant system in acid rain treated bean plant: protective role of exogenous polyamines. *Plant Sci* 2000; **151**: 59-66.

[19] Lacko-Bartošová M, Kosík T, Kobida L. Free flavonoid content and antioxidant activity of winter wheat in sustainable farming systems. *J Microbiol Biotechnol Food Sci* 2013; **2**(Special issue 1): 2099-107.

[20] Balešević-Tubić S, Malenčić D, Tatić M, Miladinović J. Influence of aging process on biochemical changes in sunflower seed. *HELIA* 2005; **28**(42): 107-14.

[21] McDonald C, Lowry L. Modification of the Folin reagent for determination of proteinase activity. *Anal Biochem* 1965; **10**: 175-86.

[22] Lowery N, Rose rough A, Randall R. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265-75.

[23] Sharma J, Singh A, Kumar R, Mittal A. Partial Purification of an alkaline protease from a new strain of Aspergillus oryzae AWT 20 and its enhanced stabilization in entrapped Ca-Alginate Beads. *Int J Microbiol* 2006; **2**(2): 1-14.

[24] Smith L, Patterson J, Walker T, Verghese M. Chemopreventive potential of sunflower seeds in a human Colon Cancer cell line. *Int J Cancer Res* 2016; **12**(1): 40-50.

[25] Achille K, Maxime A, Sabas B, Kouamé D. Detoxifying hydrogen peroxide enzymes activity in two plant species exposed to air pollution in abidjan city (côte D'ivoire). *Int J Plant, Animal Environ Sci* 2015; **5**(1): 140-5.

[26] Mehdi WA, Kasem SA, Mehde AA. Determination of several antioxidants parameters and deoxynivalenol in iraqi and other types of wheat. *Middle-East J Sci Res* 2015; **23**(7): 1420-6.

[27] Farhan L, Mustafa S, Mubder N. Effect of pregnancy on selenium, cupper, zinc and others biochemical feature. *Baghdad Sci J* 2013; **10**: 1182-9.

[28] Samieri C, Sun Q, Townsend MK, Rimm EB, Grodstein F. Dietary flavonoid intake at midlife and healthy aging in women. *Am J Clin Nutr* 2014; **100**(6): 1489-97.

[29] Abdallah A, Farid MA, Abdelwahed NA, El Shazly A. Purification, characterization and application of alkaline protease enzyme produced by Streptomyces rochei NRC24. *Int J Dev* 2013; **2**(1): 37-53.

[30] El-Khonezy M, El-Gammal E, Atwa N, El-Abd M. Partial purification and characterization of an alkaline serine protease produced

by streptomyces griseus NCRRT and its antifungal effect on Fusarium solani. *World Appl Sci J* 2015; **33**(5): 831-42.

[31] Hussain A, Manan A, Zubair H, Mirza B. Purification and characterization of alkaline proteases from Pakistan. *J Chem Soc Pak* 2010; **32**: 497-504.

[32] Deng A, Wu J, Zhang Y, Zhang G, Wen T. Purification and characterization of surfactant-stable high-alkaline protease from Bacillus sp. B001. *Biores Technol* 2010; **101**: 7100-6.

[33] El-Hadedy D, El-Gammal E, Saad MM. Alkaline protease production with immobilized cells of streptomyces flavogriseus (nrc) on various radiated matrices by entrapment technique. *Eur J Biotechnol Biosci* 2014; **2**(3): 5-16.

[34] Nadeem M, Qazi J, Syed Q, Gulsher M. Purification and characterization of an alkaline protease from Bacillus licheniformis UV-9 for detergent formulations. *Songklanakarin J Sci Technol* 2013; **35**(2): 187-95.