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

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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
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 H2O2 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 colorimetric method [19]. SOD activity [U/mg (protein)] was determined using the spectrophotometric method [20].

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 $K_m$ and $V_{max}$ 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 ($\Delta G^*$), enthalpy ($\Delta H^*$), and entropy ($\Delta S^*$) 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, H2O2, 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 $V_{max}$ at $31.45 U/mL$ and $K_m$ value of $0.142 mg/mL$.

Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pulp (Mean ± SD)</th>
<th>Peel (Mean ± SD)</th>
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<tr>
<td>Fatty acid [mg/100 gm]</td>
<td>28.59 ± 3.15</td>
<td>31.48 ± 1.85</td>
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<tr>
<td>Carbohydrate</td>
<td>2.57 ± 0.45*</td>
<td>3.47 ± 0.37</td>
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<tr>
<td>H2O2 [umol/gm. FW]</td>
<td>3.12 ± 0.46</td>
<td>3.28 ± 0.52</td>
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<tr>
<td>β-carotene [ppm]</td>
<td>6.98 ± 1.05*</td>
<td>8.22 ± 1.13</td>
</tr>
<tr>
<td>Protein [mg/mL]</td>
<td>1.57 ± 0.15</td>
<td>1.74 ± 0.25*</td>
</tr>
<tr>
<td>TPC [µg/gm]</td>
<td>2.19 ± 0.43*</td>
<td>4.68 ± 0.31</td>
</tr>
<tr>
<td>MDA [mmol/gm]</td>
<td>0.030 ± 0.003*</td>
<td>0.012 ± 0.0001</td>
</tr>
<tr>
<td>Ceruloplasmin Oxidase [mg/dL]</td>
<td>9.42 ± 1.11*</td>
<td>6.98 ± 0.79</td>
</tr>
<tr>
<td>CAT [U/mL]</td>
<td>245.2 ± 22.25**</td>
<td>216.9 ± 29.5</td>
</tr>
<tr>
<td>APX [U/mL]</td>
<td>9.56 ± 1.05**</td>
<td>14.89 ± 1.32</td>
</tr>
<tr>
<td>Free radical scavenging</td>
<td>63.14 ± 1.05*</td>
<td>69.30 ± 1.23</td>
</tr>
<tr>
<td>capacity [%]</td>
<td>555.33 ± 28.22*</td>
<td>569.41 ± 20.05</td>
</tr>
<tr>
<td>Flavonoid content [µg/g]</td>
<td>4.86 ± 1.08</td>
<td>4.52 ± 1.00</td>
</tr>
<tr>
<td>SOD [U/mg]</td>
<td>29.31 ± 0.35***</td>
<td>4.25 ± 0.10</td>
</tr>
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*P ≤ 0.05; **P ≤ 0.01 and ***P ≤ 0.001.

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

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 [24]. Plants have two methods for detoxifying the H2O2 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].

Table 2

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<tr>
<td>Enzyme Crude</td>
<td>12</td>
<td>31.10</td>
<td>373.2</td>
<td>21.9</td>
<td>17.04</td>
<td>100</td>
<td>1</td>
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<tr>
<td>Ammonium Sulfate Precipitation</td>
<td>10</td>
<td>22.5</td>
<td>225.00</td>
<td>11.55</td>
<td>19.48</td>
<td>60.29</td>
<td>1.14</td>
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<td>Dialysis</td>
<td>5</td>
<td>18.50</td>
<td>92.50</td>
<td>3.30</td>
<td>28.03</td>
<td>24.79</td>
<td>1.64</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>2</td>
<td>24.6</td>
<td>49.2</td>
<td>0.61</td>
<td>80.66</td>
<td>13.18</td>
<td>4.73</td>
</tr>
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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.

Figure 4. Arrhenius plot.

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, lipoxygenase, 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 anti-
oxidant properties, such as CAT and SOD [28]. The present study shows peak in sunflower are strong radical scavengers and can be con-
sidered 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]. 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].

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

[21] McDonald C, Lowry L. Modification of the Folin reagent for deter-


