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Evaluation a Dozen Biochemical Indexes of the Catalase Enzyme in Safflower (*Carthamus tinctorius* L.)

[1]Shima Nosrati, [1]Maziar Ghandian Zanjan and [2]Davood Eradatmand Asli

[1]*Islamic Azad University, Department of Agronomy and Plant Breeding, Agriculture Faculty, Box.39187-366. Saveh, Iran.*
[2]*Payame Noor University, Department of Agriculture, Agriculture Faculty, Box. 19395-3697. Tehran, IRAN.*

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A B S T R A C T

Safflower (*Carthamus tinctorius* L.) includes species of high medicinal and food value. Due to the high resistance of safflower to stressful environmental conditions, scientists use the plant as a model to study and understand the defense mechanisms against environmental stresses. According to antioxidant enzyme systems as one of the most important factors in increasing the resistance of plants to environmental stresses, in this study, some biochemical properties of the catalase enzyme in Safflower (*Carthamus tinctorius* L.cv. I L-111) and its sensitivity were determined to both inhibitors azide and cyanide. Safflower plants were grown hydroponically in perlite growing media for 40 days. Catalase (EC 1.11.1.6) was extracted from leaves of the plant by phosphate buffer pH=7.2, 0.1M. Study of different pH effects, various concentrations of substrate, azide and cyanide inhibitors, effect of temperature on catalase activity of safflower and also checking out the non-denaturant electrophoresis gel from safflower extract confirmed, there are at least two isoenzyme of catalase, respectively, with the optimum pH 6.5 and 8.5 at the plant. Active isoenzyme in pH 8.5 in comparison with active isoenzyme in pH 6.5 is more resistant to temperature **.** Inhibitory effect on catalase isoenzymes showed that the isoenzyme in optimum pH 8.5 in comparison with isoenzyme with the optimum pH 6.5 is more sensitive respectively 4.6 times to cyanide ions and 2.6 times to azide.

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INTRODUCTION

Safflower scientific name *Carthamus tinctorius* L. belongs to Rutaceae. Plant Origin is in Saudi Arabia and its culture in Iran and countries like India, Pakistan and Afghanistan is great antiquity. Edible oils are made by safflower seeds and from their flowers are used to make flavored foods and color product. Iran because of abundant cultivated and wild species the plants is one of the most important Safflower genetic reserves (Zohary and Hopf, 2000)**.** In general, the plants through specific mechanisms of physiology and biochemistry will counter with various environmental stresses. Mechanisms of resistance in some of stresses as an internal communication planning and result of this are a harmonious and complex planning. In terms of stress, lack of the balance between energy absorption and its consumption produced various species of active oxygen and plant inability to inhibit by photosynthetic organs, which ultimately leads to injury oxidative (Chaudiere and Ferrari-Lliou, 1999). Different mechanisms found to counter active oxygen species in living organisms that can be pointed one of the most important in name of antioxidant enzyme systems. This system includes enzymes such as dismutase superoxide, catalase and peroxidase. Dismutase superoxide enzymes reduced toxicity of superoxide anion by converting O_2 to H_2O_2 and then hydrogen peroxide has accumulated in the environment, has detoxified by group of enzymes called hydro peroxides. Hydro peroxides of the following three different groups of the enzyme have formed called catalases, two functional catalase peroxides and peroxides (Chaudiere and Ferrari- Lliou, 1999). Catalase (H_2O_2:H_2O_2 reductase oxide EC1.11.1.6) is an enzyme which was found in all organisms including; plant, animal and aerobic micro-organism cells and also as one of the most important antioxidant enzymes play considerable role in reducing hydrogen peroxide by decomposition of H_2O_2 to water and oxygen (Bloch et al., 2007; Preston et al, 2001; Scandalios *et al*., 1997). In pplants, catalase enzyme are in organelles called peroxisome and has important role in scanning of H_2O_2 that has produced by processes like oxidation of fatty acids –β, oxidation during light respiration and electrons transmission chain of mitochondria respiratory (Scandalios *et al*. 1997). In addition, the role of catalase in the defense system and the process of aging in plants also have been proven (Mura et al. 2007). Biochemical properties of the catalase enzyme has been investigated in many plants such as saffron, mustard, spinach, cotton, parsley, wheat, sunflower, corn, castor beans, tobacco and kohlrabi and has been found that this enzyme in most listed plants has several

Corresponding Author: Maziar Ghandian Zanjan, Islamic Azad University, Department of Agronomy and Plant Breeding, Agriculture Faculty, Box.39187-366. Saveh, Iran.
E-mail: maziyar.ghandi@gmail.com

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isoenzymes (Tayefi-Nasrabadi, 2008; Mullen and Gifford, 1993; Garcia et al., 2000 ; Keyhani et al., 2002). There is no safflower descriptions in books and world botanical scientific resources, particular basic information in physiology and biochemistry of the plant. The purpose of this study was to investigate some biochemical properties of catalase enzyme such as number of isoenzymes, inhibitors of azide effect and cyanide, kinetic parameters (Km and Vmax) catalytic efficiency, and thermal stability in crude extracts of safflower.

MATERIALS AND METHODS

All chemicals used for electrophoresis were purchased from Sigma Corporation (Sigma Chem.Co). Other materials used in this study were prepared from Merck.

Preparation of plant samples:

The safflower seed is produced from Karaj Seeds and Plants Improvement Institute and in form of certain repetitions were cultured in perlite hydroponic conditions. Plants were growing with Hoagland solution for 40 days. Preparation of extract, 1 g of safflower leaves with 3ml of phosphate buffer (0.1 Molar, 7.2 pH) containing 1 mm EDTA, 2 percent Polyvinylpyrrolidone and also 0.02 percent P.M.S.F (To increase the stability of catalase) was homogenized by homogenizer machine. Then the homogenized mixture immediately was centrifuged at 20,000 revolutions per minute for 20 min at 4 ° C. The resulting supernatant was used for subsequent measurements. To measure total protein was used by Lowry's method (Lowry et al., 1951).

Measurements catalase activity:

Catalase enzyme activity was measured by measuring the reduction in absorbance due to the decomposition of hydrogen peroxide substrate at a wavelength of 240 nm considering the molar absorption coefficient 27 $Cm^{-1}.M^{-2}$ Was determined by spectrophotometric method (Obinger et al., 1997). The test solution with 3ml final volume, containing 100 µl extract, citrate - phosphate – borate buffer (0.1M with relevant pH). 50 µl H_2O_2 with final concentration 0.5 – 38 mm is used (for calculate Km and Vmax) or 10mm (for other measurements). One unit catalase enzyme activity is determined as required enzyme for decomposition of 1µm hydrogen peroxide substrate in one minute. All measurements were performed three times and the average results were reported.

Thermal stability testing:

In order to measure the thermal stability of the catalase enzyme, an appropriate amount of extract was incubated (in the piping Eppendorf 1.5ml) at the temperature of between 25-70ºC in thermo-static bath system, and then extract at specified time (5-60 min) was removed from bath system and during 5 min placed the ice bath. The catalase activity of the extract was determined in lab temperature in accordance with the above procedure. The catalase activity of the extract was considered at the temperature 25 ºC as 100% activity (Tayefi-Nasrabadi, 2008).

Catalase activity in the presence of azide and cyanide inhibitors:

To study the effect of each Azide and Cyanide inhibitors on the activity of safflower extract catalase, varying amounts of each of these inhibitors were added separately to the reaction mixture containing the extract and hydrogen peroxide was added to initiate the reaction.

Catalase activity of extracts by electrophoresis:

In order to determine the minimum number of catalase isoenzymes safflower extracts from natural electrophoresis (non-denaturant) with polyacrylamide gel and stained by enzyme activity was performed according to Woodbury method (Woodbury *et al*., 1971). First, the gel was incubated for 10 min in a solution of 0.003percent hydrogen peroxide, then was washed with twice distilled water by the $FeCl_3$ solution (one percent) and stained by $K_3Fe(CN_6)$ (one percent) for 10 min.

RESULTS AND DISCUSSION

This study aimed at identifying different type of catalase isoenzymes in this species were effect of different pH (pH profile production), different concentrations of substrate (Km and Vmax), azide inhibitors and cyanide as well as the effect of temperature (stability of temperature) on activity of safflower catalase enzyme.

Figure1 indicates pH profile, in other word; changes in CAT activity extract safflower in the presence of hydrogen peroxide substrate (10 mM concentration) at different pH. The results from this figure shows; first we can observe activity of catalase in this plant in pH wide range (from 5 to 11.5). Second pH profile that has been obtained has two indicator peaks and distinct are respectively in pH 6.5 and 8.5. According to Fullbrook's theory (Fullbrook, 1996) existence of several different optimal pH of a solution for an enzymatic activity, indicating separate isoenzymes for specific enzyme in solution is quite vital (Schulz, 1994). According to this

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theory, it can be predicted that the safflower probably contains at least two isoenzymes of catalase that has respectively optimal pH 6.5 and 8.5. For further investigations, the values of Vmax, Km, catalytic efficiency (Vmax / Km) were measured in both catalase enzyme of safflower extract and its sensitivity to azide inhibitors and cyanide optimal pH (Table 1 and Figure 2). By examining the results in Table 1 observed the values of Vmax and Km derived from CAT activity in extract safflower are absolutely different in two optimum pH 6.5 and 8.5. So that the Km active CAT in pH=8.5 is exactly 2 times the Km active CAT in pH=6.5. Also values of Vmax active CAT in pH=8.5 almost 2 times the Vmax active CAT in pH=6.5 is obtained. Figure 2 it showed effect of azide and cyanide inhibitors on catalytic activity of safflower in two optimal pH. By analyzing above figure and calculation of IC_{50} values for each inhibitors, it is observed that isoenzyme with optimum pH 8.5 in comparison with optimum pH of isoenzyme is 6.5 to cyanide ion 4.6 times and to azide ion 2.6 times more sensitive.

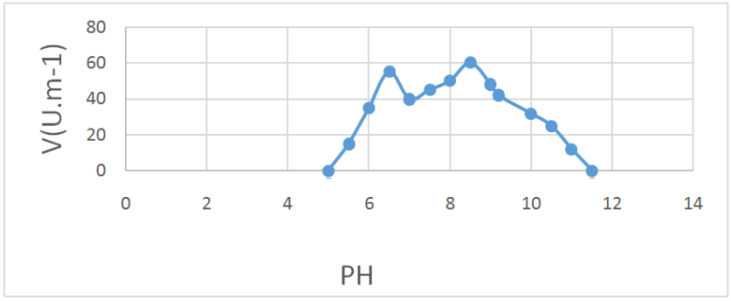

Fig. 1: pH dependency of catalase activity in leaves extract of safflower (cv. IL-111).

Table 1: Kinetic parameters and sensitivity to azide cyanide of the catalase activity in safflower (cv.IL-111) leaves extract at two pH optima.

pH optima	Km (mM)	Vmax (U/ml)	Vmax/Km	IC_{50} for azide	IC_{50} for cyanide(µM)
6.5	3.5	56.49	16.14	250	125
8.5	7	110.22	15.74	95	27

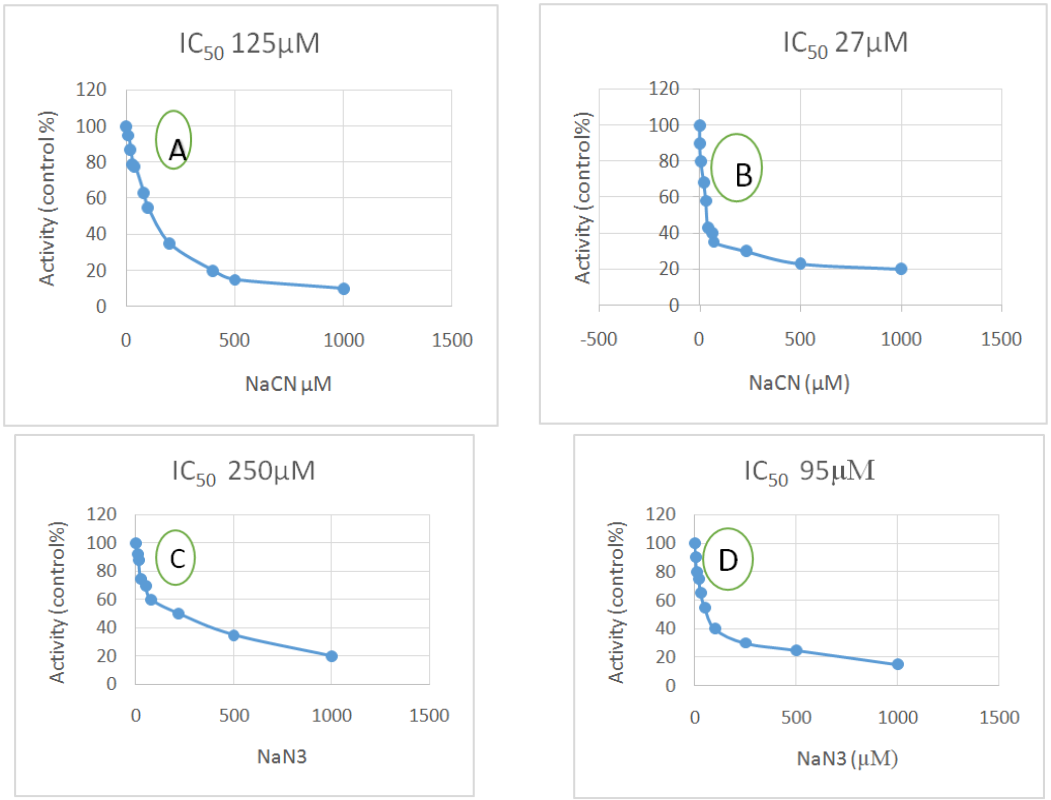

Fig. 2: Inhibition by cyanide against IL-111 catalase at pHs 6.5 (A) and 8.5 (B). Inhibition by azide against IL-111 catalase at pH 6.5 (C) and 8.5 (D).

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Thermal stability of safflower plant catalase isoenzymes is shown in Figure 3. By observing the above figure it can be concluded that the active isoenzyme pH 6.5 in comparison with isoenzymes active in the pH 8.5 is more sensitive about temperature, so that after 60 minutes incubation at 70 °C, its activity was observed 20% while another activity of isoenzyme was under the same conditions about 40% of their shows. The results of the above shows that the two isoenzymes of active catalase in safflower have kinetic properties completely separated. Distinct kinetic properties for each of catalase enzyme in Safflower extract indicating the presence of separated metabolic functions for each of these isoenzymes in the plant. For final determination, minimum order of catalase isoenzymes was used in safflower, from the natural Polyacrylamide electrophoresis gel (non-denaturant) and stained by catalase activity (Figure 4). It can be seen in Figure 4, after distinctly gel sainting, existence of two distinct bands in the gel is observed existence of two distinct bands indicates that at least two catalase isoenzyme have been in safflower that the result is confirmation of previous results from the analysis of the kinetic data of the safflower plant catalase.

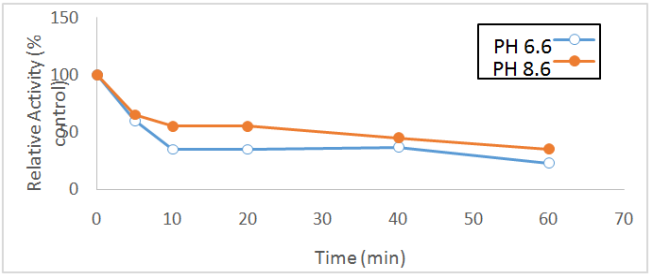

Fig. 3: Thermal tability of IL-111 catalase at pHs 6.5 (o) and 8.5 (·). Extract was incubated for various time intervals (1 - 60 min) at 70 °C.

Fig. 4: Non-denaturing PAGE of extract stained for catalase activity. Bands up and down presente different catalase isoforms.

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