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Full Length Research Paper

Catalytic properties of three catalases from Kohlrabi (*Brassica oleracea gongylodes*)

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Catalase (EC 1.11.1.6) was extracted from kohlrabi bulbs (*Brassica oleracea* g*ongylodes*) with 0.05 M phosphate buffer, pH 7.0. On the basis of kinetic studies and activity stain for catalase, only three isoenzymes of catalases were detected in kohlrabi bulbs extract with pH optima at 4.5, 6.5 and 10. Highest catalytic efficiency (V_{max}/K_m) value was found for isoenzyme active at pH 6.5. Isoenzyme with pH optima at 4.5 was very sensitive to azide and more resistant to cyanide in comparison to other two isoenzymes active in kohlrabi bulbs extract. Substrate inhibition was found only for the isoenzyme active at pH 4.5. Heat inactivation studies showed a decrease in catalases activity at temperatures above 50, 60 and 70°C for isoenzymes active at pH 6.5, 10 and 4.5, respectively.

Key words: Kohlrabi, catalase, crude extract, kinetic, isoenzyme, thermal stability.

INTRODUCTION

A group of enzymes designated as hydroperoxidases, prevent H_2O_2 accumulation in living organism. This group includes three sub-classes of proteins: catalases, the bi-functional catalase-peroxidases, and peroxidases (Chaudiere and Ferrari, 1999; Oztürk et al., 2007). Catalase (EC 1.11.1.6), which degrades H_2O_2 into water and oxygen, is one of the major antioxidant enzymes (Scandalios et al., 1997). It is one of the first enzymes to be purified and crystallized and has gained a lot of attention in recent years because of its link to cancer, diabetes, and aging in humans and animals (Melov et al., 2000; Preston et al., 2001; Turdi et al., 2007). In plants, catalase scavenges H_2O_2 generated during mitochondrial electron transport, β-oxidation of the fatty acids, and most importantly photorespiratory oxidation (Scandalios et al., 1997). Accumulating evidence indicates that catalase plays an important role in plant defense, aging, and senescence (Mura et al., 2007; Conrath et al., 1995). Catalase has been characterized from many prokaryote and eukaryote organisms so far. Catalase has been reported to exist in multiple forms in many higher plants, such as saffron, mustard, spinach, cotton, wheat, sun-flower, maize, castor bean, loblolly pine, and tobacco (Mullen and Gifford 1993; Garcia et al., 2000; Keyhani et al., 2002).

Kohlrabi (*Brassica oleracea* g*ongylodes*) is a low, stout cultivar of the cabbage that will grow almost anywhere and used as a food and also as a traditional medicine. The plant is carefully described in botanical books, but there is no available information on the basic aspects of its physiology and biochemistry. The purpose of this research was to investigate and characterize catalase activity in kohlrabi bulbs.

MATERIALS AND METHODS

Chemicals

Chemicals for electrophoresis and protein assay reagent were purchased from Sigma Chem. Co. All the other chemicals used in this work were obtained from Merck and were of reagent grade.

Plant materials

Kohlrabi bulbs was collected during summer from the North-west of Iran (Tabriz) and stored at 4°C. For the preparation of the crude extract, 100 g of Kohlrabi bulbs was finely chopped and homo-genized with 100 ml of 0.05 M phosphate buffer, pH 7.0 containing 0.1% polyvinylpyrrolidone (for stabilizing the extract), in a Waring Blender for 5 min. The homogenate rapidly filtered through a layer of cheesecloth, and centrifuged at 20,000 g for 30 min at 4°C. A clear, transparent supernatant termed “crude extract” was obtained and used for our studies. Protein concentrations were determined by the method of Lowry et al. (1951), using crystalline bovine serum albumin as standard.

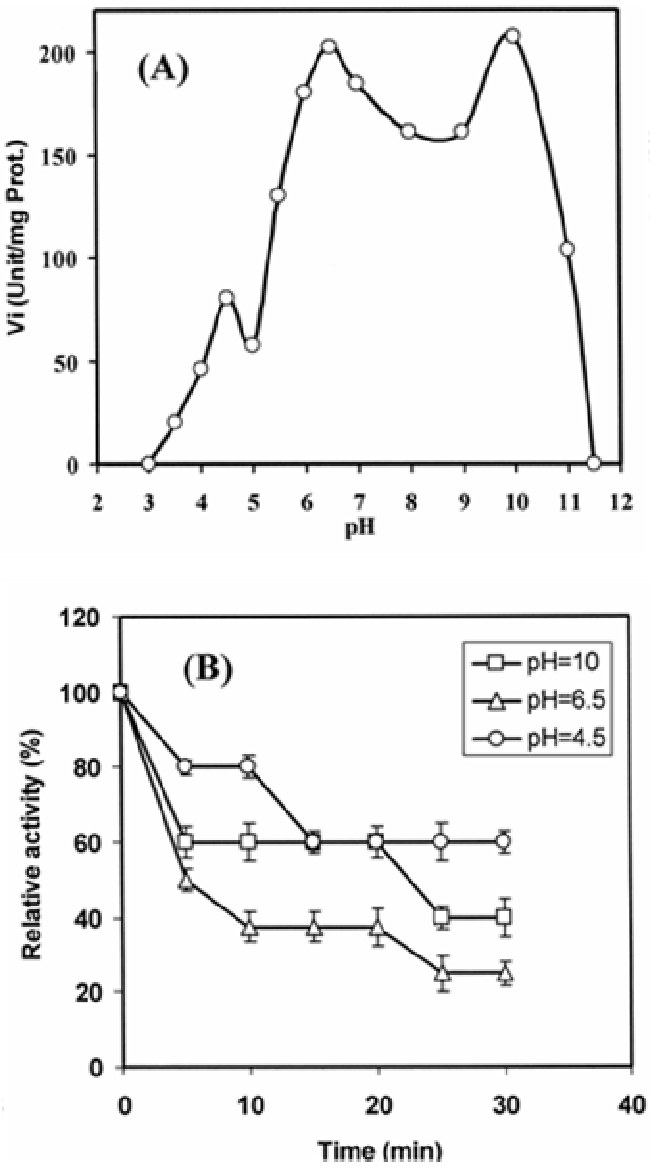

Figure 1. (*A*) pH dependency of catalase activity in kohlrabi bulbs. (*B*) Thermal stability of kohlrabi catalase at pH 4.5, 6.5 and 10. Extract was incubated for various time intervals (5 - 30 min) at 70°C and then tested at room temperature, at given pH, after brief cooling in ice. The activity was measured at 25°C, was taken as 100%.

Catalase activity assay

Catalase activity was measured by following the dismutation of H_2O_2 spectrophotometrically using an extinction coefficient for H_2O_2 at 240 nm of 27 $M^{-1}.cm^{-1}$ (Obinger et al., 1997). Assays were carried out at room temperature (~22 - 25°C), with an UNICO UV-2100 PC spectrophotometer. 100 µl extract (965 µg protein/ml) were added to 0.1 M citrate-phosphate-borate buffer, at given pH, containing H_2O_2 in final concentration of 0.5 to 38 mM (for K_m, V_{max} determinations) or 10 mM (for all other assays); the reaction mixture total volume was 3 ml. Reaction velocity was computed from linear slopes of absorbance-time curve (Wong et al., 1971). One unit of catalase activity was defined as the amount of enzyme necessary for reducing 1µmol of H_2O_2 per minute.

Thermal stability assay

Thermal stability of kohlrabi catalases were studied by incubating aliquots of extract at various temperatures (25 - 70°C) up to 30 min in a thermostatic water bath and measuring their activity at room temperature after brief cooling in ice. The incubation was carried out in sealed vials to prevent change of volume of the sample and, hence, the enzyme concentration due to evaporation. The activity was measured at 25°C, was taken as 100%, and activities which were measured (35 - 70°C) were compared with the activity measured at 25°C.

Activity staining for catalase

Catalase activity in native PAGE gels was determined as described by Woodbury et al. (1971). Gels were incubated in 0.003% H_2O_2 for 10 min and developed in a 1% (w/v) $FeCl_3$ and 1% $K_3Fe(CN_6)$ (w/v)) solution for 10 min.

RESULTS AND DISCUSSION

Kinetic studies for identification of various types of catalases in kohlrabi bulbs were based on the following criteria as suggested by Schulz (1994) and Fullbrook (1996): (i) variation in activity as a function of pH; (ii) variation in activity as a function of substrate concentration (K_m and V_{max}); (iii) effect of inhibitors on activity (differential effects of azide and cyanide); (iv) effect of temperature on activity.

Figure 1A shows the H_2O_2 dismutation by kohlrabi bulbs extract in the presence of 10 mM H_2O_2 at different pH, ranging from 3.5 to 11.0 expressed as units of enzyme per mg extract protein. Three peaks were found, respectively at pH 4.5, 6.5 and 10.0. As suggested by Fullbrook (1996) the presence of various pH optima indicates the presence of distinct enzymes. Table 1 shows the K_m, V_{max}, and catalytic efficiency (K_m/V_{max}, calculated per mg extract protein) at the respective pH. The K_m, V_{max} and catalytic efficiency were different for all three pH examined. Their distinctive kinetic properties suggested possible different metabolic roles for each isoenzyme in this plant.

Effect of substrate concentration (hydrogen peroxide) on activity alteration at three pH optima showed that only catalase activity at pH 4.5 was inhibited by hydrogen peroxide at concentration above 5 mM. At pH 4.5, the maximum rate was 299.27 µM.min^{-1} for a substrate concentration of 5 mM; thereafter, substrate inhibition was observed, and for 15 mM H_2O_2, the activity was reduced to 44.89 µM.min^{-1} (results not shown).

The effect of two inhibitors, namely azide (NaN_3) and cyanide (NaCN) on catalase activity in kohlrabi bulbs extract were also studied. As shown in Table 1, catalase activity at pH 4.5 in comparison to other two pH optima has a highest and lowest sensitivity to azide and cyanide,

Table 1. Kinetic parameters and sensitivity to azide and cyanide of the catalase activity in kohlrabi bulbs extract at three pH optima.

pH optima	K_m (mM)	$V_{max}^{(*)}$ (Unit/ml)	$V_{max}/K_m^{(*)}$	IC_{50} values for azide (µM)	IC_{50} values for cyanide (µM)
4.5	1.0	299.27	299.27	10	12000
6.5	0.64	207.9	324.84	75	200
10.0	13.4	598.64	44.67	1000	2000

(*)Calculated per mg extract protein.

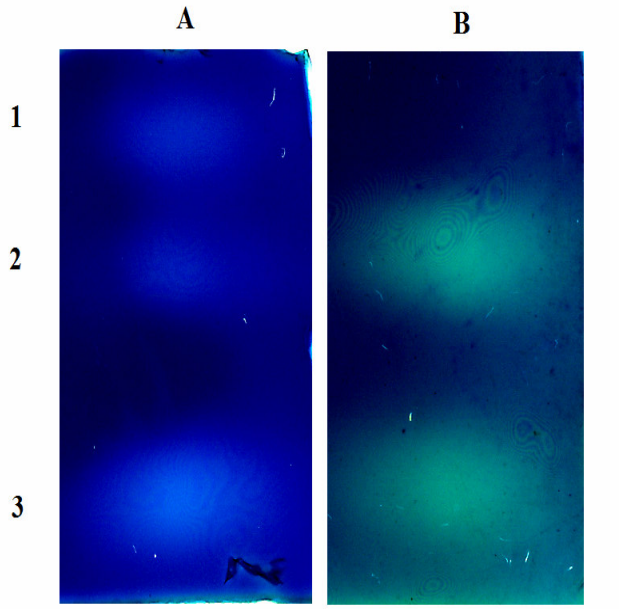

Figure 2. Non-denaturing PAGE of extract stained for catalase activity. Lane A, 20 µl extract; Lane B, 20 µl extract + azide (100 µM).

respectively. Order of sensitivity of catalase activity to azide at three pH optima was pH 4.5 > pH 6.5 > pH 10, with IC_{50} of 10, 75 and 1000 µM, respectively. IC_{50} value was found for cyanide at pH 4.5 was 1200 times greater than to azide, showing that catalase activity at pH 4.5 was remarkably insensitive to cyanide in comparison to other two pH optima. The results confirming the presence of at least three distinctive isoenzymes of catalase in kohlrabi bulbs extract, as suggested by the three pH optima.

The Thermal stability of the catalase activity in kohlrabi bulbs extract, presented as the residual percentage activity was shown in Figure 1B. As shown in this figure, after heating the extract at 70°C for 30 min, catalase activity lost about 40, 60 and 75% of the original activity at pH 4.5, 10 and 6.5, respectively. When the extract was incubated at specified temperature (25 - 70°C) for various length of time (5 - 30 min) then tested at room temperature, a decrease in catalase activity was found at temperature above 50, 60 and 70°C for pHs of 6.5, 10 and 4.5, respectively (results not shown). Therefore, the order of thermostability of three catalases isoenzymes from kohlrabi bulbs extract at three pH optima was pH 4.5 > pH 10 > pH 6.5.

The presence of three isoenzymes was confirmed by when the extract was submitted to non-denaturating PAGE, followed by activity staining for catalase (Lane A in Figure 2). After preincubation of the extract with 100 µM azide, only two bands (bands 2 and 3) were visualized on the gel (Lane B in Figure 2). This result suggested that band 1 could be corresponded to the catalase isoenzymes with pH optima at 4.5. Since catalase activity at pH 4.5 was more sensitive to azide (IC_{50} = 10 µM) in comparison to other two pH optima (Table 1).

Conclusion

Three isoenzymes of catalases were detected in kohlrabi bulbs extract with pH optima at 4.5, 6.5 and 10. Isoenzyme active at pH 4.5 have the highest and lowest sensitivity to azide and cyanide, respectively. The order of catalytic efficiency (V_{max}/K_m, calculated per mg extract protein) for three isoenzymes of catalase at three pH optima was pH 6.5 > pH 4.5 > pH 10. Substrate inhibition was found only for the isoenzyme active at pH 4.5. The order of temperature stability of three catalases at three pH optima was pH 4.5 > pH 10 > pH 6.5.

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