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PURIFICATION AND CHARACTERIZATION OF AN ANTIOXIDANT METALLOENZYME FROM LOCAL TOMATO VARIETY.

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ABSTRACT

The isolation, purification and characterization of an antioxidant enzyme superoxide dismutase (SOD), from local tomato variety, has been reported in this study. Whole tomato extracts from different ripening stages were prepared in chilled 0.01M citrate phosphate buffer (pH 7.0) containing protease inhibitor. SOD activity along with total protein content at various stages of maturity and ripening were correlated. The "rotten" tomato sample, showing highest specific activity i.e. 140 IU/mg was selected for further purification and was subjected to 80% ammonium sulfate precipitation. Precipitated proteins were dialyzed and loaded to sephadex G-75 column for gel filtration chromatography. The enzyme was purified up to 6 fold with a final specific activity of 825 IU/mg with a 40.4 % yield. The PAGE analysis of purified sample under denaturing conditions showed a single band of 16 KDa. The purified enzyme was thermally stable under mesophilic conditions i.e. up to 40°C and was most stable between acidic to neutral pH values 5.0-7.0. Based on atomic absorption spectroscopic analysis and sensitivity to inhibitors such as urea, H_2O_2, chloroform-ethanol and KCN, the enzyme was found to be Fe-SOD.

INDEXING TERMS/KEYWORDS

SOD, ripening, rotten, purification, sensitivity.

ACADEMIC DISCIPLINE AND SUB-DISCIPLINES

Life Sciences

SUBJECT CLASSIFICATION

Biochemistry

TYPE (METHOD/APPROACH)

Research article (protein purification steps: Ammonium sulphate precipitation, dialysis, gel filtration chromatography. Then characterization of purified enzyme by analyzing optimal pH and temperature, metal ion analysis using atomic absorption technique and effect of inhibitors.)

enzymatic mechanisms, help to reduce the toxicity of these superoxide anions. Among enzymatic mechanisms, superoxide dismutase (SOD) enzymes have proved to be very efficient [6]. Epecially during different stages of plant growth, development, senescence, and even pre- and post-harvest fruit ripening, SODs protect cells and tissues from damaging effect of superoxide anion by removing them before they undergo Fenton's reaction i.e. reaction of superoxide anion with cellular H_2O_2 to form highly reactive hydroxyl radicals (OH^-)[7,8].

Based on the presence of prosthetic metal group at active site, plants possess three types of SODs: Cu/Zn SOD [9], Mn SOD [10], and Fe SOD [11]. The Cu/Zn SOD is the most studied form and is usually present in higher plants where

it is mainly located in plastids and cytosol while Mn-SOD is predominantly present in the mitochondrial matrix. The Fe-SOD is located in chloroplasts of only some dicotyledonous plant species but not all plant species [12].

Presently, SODs are gaining clinical importance because of their anti-inflammatory effect in humans [13]. They tend to protect many cell types from free radical damage that could cause ischemic tissue. They prevent DNA damage, lipid peroxidation, protein denaturation, and many other forms of progressive cell degradation. SODs are also used in cosmetic products to reduce free radical damage to skin, for example to reduce the risk of fibrosis after radiation therapy for breast cancer [14]. These metal reductases also find application in resisting other types of radiations, tumors and stress along with delaying ageing [10]. Because of these properties many pharmaceutical and cosmetic companies have also shown interest in SOD enzyme to be used in their products [15].

The main objective of the present research was to isolate SOD enzyme from local tomato variety as it is sufficiently available and has short growth time. Moreover, possible correlation between total protein and superoxide dismutase (SOD) levels at different ripening stages was investigated. The stage showing maximum SOD activity was selected and subjected to purification and characterization. The purified SOD could be used as an anti-aging and anti-inflammatory agent in medicines and ointments.

MATERIALS AND METHODS

All operations in the present study were carried out at 0-4°C.

Sample collection

Tomato samples, at nine different ripening stages, were harvested from vegetable research farm of University of the Punjab, Lahore. The redness of fruit and firmness of skin were used as an index of maturity. Samples were then immediately stored in ice for further experiments.

Extract preparation

The samples were individually rinsed with distilled water, diced and homogenized at 4˚C in 0.01M citrate phosphate buffer (pH 7.0) containing protease inhibitor (phenylmethane sulfonyl flouride). Cells were further disrupted by sonication, (SANAYO Soniprep 150 sonifier) followed by centrifugation at 10,000 g for 10 minutes at 4˚C (Shimadzu centrifuge 3-30 K). The supernatants were then used as the crude enzyme extracts.

Enzyme assay

SOD activity was determined using Real Quant Superoxide Dismutase assay kit following the manufacturer's instruction. The kit utilized a dye that produced a water soluble formazan upon reduction with superoxide anion. The rate of reduction with superoxide anion was linearly related to the xanthine oxidase (XO) activity and was inhibited by SOD. Higher the SOD activity, lesser was the formation of formazan and lower was the OD value at 470 nm. One unit SOD inhibits the rate of reduction of cytochrome c by 50% in a coupled system using xanthine and xanthine oxidase under optimized conditions of temperature and pH.

Total protein estimation

The total protein concentration was estimated in all crude extracts with Bradford assay using BSA as a standard [16].

Assay pH and temperature optimization

The pH value of the crude enzyme solution (stage 9) was adjusted to 4, 5, 6, 7 and 8 with the following buffers: citrate phosphate (pH range 3.4-7.0) and Tris-HCl (pH range 7.5-9.0) and then incubated at 37 °C for 30 minutes. The enzyme activity was then assayed for optimal assay pH.

To study the optimal assay temperature, crude enzyme solution (stage 9) was incubated for 30 min at 25, 37, 45, 55 and 70°C in buffer of optimized pH and was assayed for its maximum activity.

Enzyme purification

Finely powdered ammonium sulfate was added to 80% saturation. The mixture was left overnight at 4° with constant stirring, followed by centrifugation at 13000 g for 10 minutes at 4˚C. The precipitated protein pallet was dissolved in buffer and dialyzed overnight against the same buffer at 4°C with constant stirring. 1 ml of the dialyzed sample was loaded on Sephadex G-75 column (0.8 ×15 cm) and equilibrated with 100 ml of 0.01M citrate phosphate buffer pH 5.0. 2 ml fractions were collected, using same buffer for elution and each fraction was then assayed for enzyme activity. Purified enzyme was then subjected to SDS-PAGE for size determination using 10% acrylamide gels (mini protein electrophoresis cell, BioRad), following the protocol described by Laemmli [17].

Thermal and pH stability of purified SOD

The thermostability of purified enzyme was investigated at 4, 25, 37, 45, 55 and 70 °C for 30 minutes in 0.01M citrate phosphate buffer pH 5. Residual activity was then assayed.

The purified enzyme's stability towards pH was examined at pH range 3.0-9.0 using Glycine-HCl (pH range 2.0-3.0), citrate phosphate (pH range 3.4-7.0) and Tris-HCl (pH range 7.5-9.0) buffer systems for 30 minutes at 37°C. Residual activity was then assayed.

Effect of selective inhibitors on SOD activity

10 mM potassium cyanide, 10 mM urea, 1.5% (V/V) hydrogen peroxide and 2:3 (V/V) chloroform -ethanol were tested as selective inhibitors for SOD activity. An enzyme solution containing each compound was incubated in 0.01 M citrate phosphate buffer pH 5 at 37°C. The SOD activity was then determined.

Metal ion analysis

Metal content of the purified enzyme, after exhaustive dialysis to remove traces of any contaminating metals, was analyzed by atomic absorption spectrometry with a Perkin Elmer AAnalyst 100 apparatus equipped with Acetylene -Air (1:3) and a slit width of 0.7nm. Standard solutions of iron, copper, manganese and zinc ranging from 1-5 ppm were used.

RESULTS

Superoxide dismutases showing strong antioxidant properties, protect normal cells from reactive oxygen species (ROS) [18]. The crude extracts of tomato samples from nine different ripening stages were prepared and were analyzed further as follows:

Total protein concentration determination and SOD activity assay

Determination of total protein concentration along with SOD activity was performed in all nine crude tomato extracts. There was a constant drop in total protein content with the progression in ripening stages (Figure 1) while the SOD enzyme activity was higher in immature green stage (stage 1), reduced in stages from 2 to 6, increased again in stages 7 and 8 and was maximum in the rotten tomato sample (stage 9). Hence, stage 9 sample was chosen for further studies.

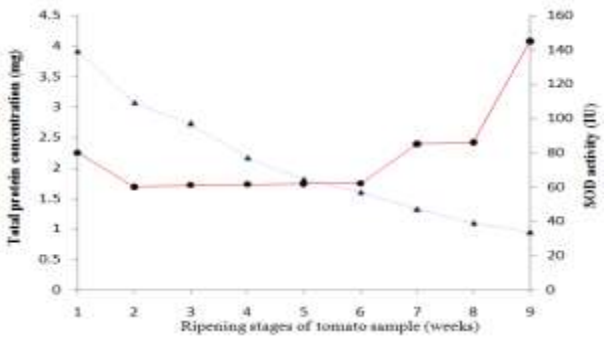

Figure1: Total protein content and SOD activity in tomato samples at different ripening stages

Assay pH and temperature optimization

The optimal assay pH value was determined by incubating crude enzyme sample (stage 9) at pH 4, 5, 6, 7 and 8 with the following buffers: citrate phosphate (pH range 3.4-7.0) and Tris-HCl (pH range 7.5-9.0) for 30 minutes at 37°C. Maximum activity was found to be 134 IU at pH 5 which was the optimal pH for the SOD enzyme (Figure 2).

The optimal assay temperature was studied by incubating the sample for 30 min at 25, 37, 45, 55 and 70°C in citrate phosphate buffer (0.01 M, pH 5.0). Maximum activity of the sample was found to be 143 IU at 37^{o}C which was the optimal temperature for SOD enzyme (Figure 2).

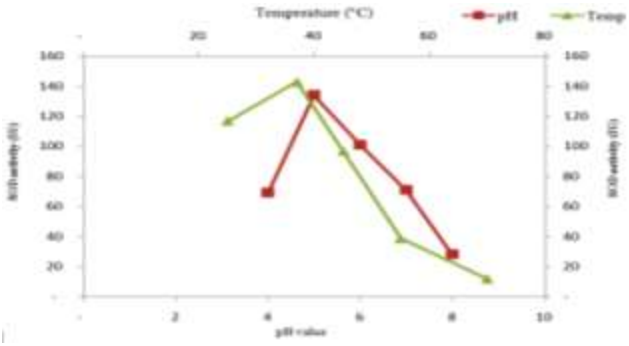

Figure 2: Effect of pH and temperature on SOD activity

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Enzyme purification

SOD was extracted from rotten tomato sample with citrate phosphate buffer pH 5 and purified through ammonium sulfate precipitation, dialysis and gel filtration chromatography. Results showed that 140 IU/mg specific activity was present in the crude extract that rose to 231.2 IU/mg after dialysis. It further increased up to 825 IU/mg after purification through gel filtration chromatography using Sephadex G-75 resin. Around 6-fold purification was achieved (Table 1).

Table1. Total protein concentration in the crude sample and after different purification steps.

Purification	Volume (ml)	Total protein(mg)	Total SOD activity (IU)	Specific SOD activity (IU/mg)	Purification (fold)	Yield (%)
Crude extract	50	1.75	245	140	1.0	100
Dialyzed sample	3.0	0.80	185	231.2	1.65	75.5
Sephadex G-75	2.0	0.12	99	825	5.89	40.4

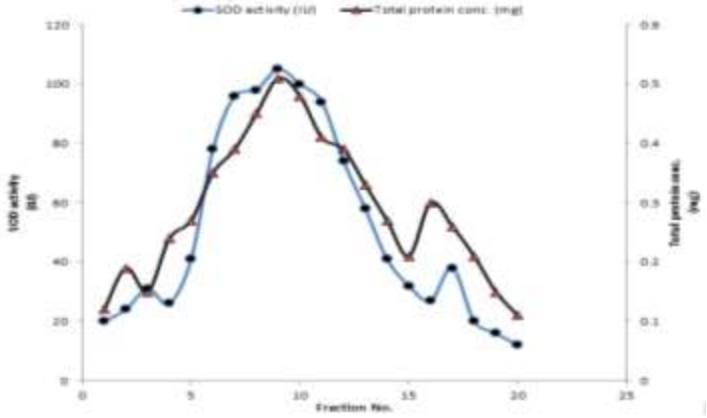

Figure 3: Elution profile of tomato SOD by gel filtration chromatography in the Sephadex G-75

The active fractions (Figure 3) were pooled, concentrated by lyophilization using freeze dryer (CHRIST ALPHA 1-4, Type: 100400, K.W 1.3) and the pallet was appropriately diluted to be subjected to SDS-PAGE under denaturing conditions that displayed the molecular weight of purified sample to be 16 kDa (Figure 4).

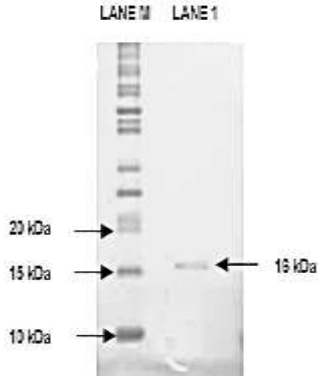

Figure 4: SDS-PAGE analysis after gel filtration chromatography

Lane M: protein ladder;

Lane 1: 16 kDa band of purified SOD

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Thermal stability of purified SOD

The thermal stability of the purified enzyme was examined at standard assay conditions after 30 minutes of incubation at 37°C, using 0.01 M citrate phosphate buffer pH 5. As can be seen in Figure 5 the activity of purified SOD remained relatively stable upto 99.87% till 37°C but was rapidly reduced to 49.5% at 45°C and got inactived below 70°C.

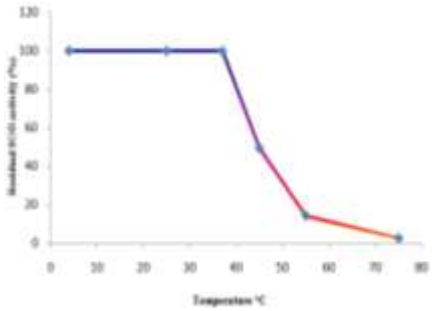

Figure 5: Thermal stability profile of Tomato SOD activity

pH stability of purified SOD

The pH dependence of the enzyme stability was measured from the residual activity at standard activity assay conditions. The purified enzyme was investigated at pH 3, 4, 5, 6, 7, 8 and 9 using Glycine-HCl (pH range 2-3), citrate phosphate (pH range 3.4-7.0) and Tris-HCl (pH range 7.5-9.0) buffer systems, at 37°C after 30 minutes. As visible in Figure 6, the activity of purified SOD remained comparatively stable between pH 5-7 but was merely active outside this range.

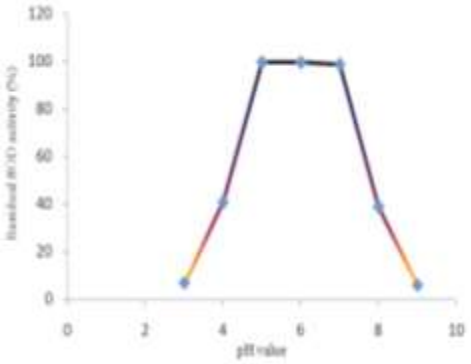

Figure 6: Effect of pH on Tomato SOD stability

Effect of inhibitors

Effect of urea, H_2O_2, chloroform-ethanol and KCN were studied on the purified SOD activity. Figure 7 show that enzyme was sensitive to urea, H_2O_2 and chloroform-ethanol while KCN had no effect on the SOD stability.

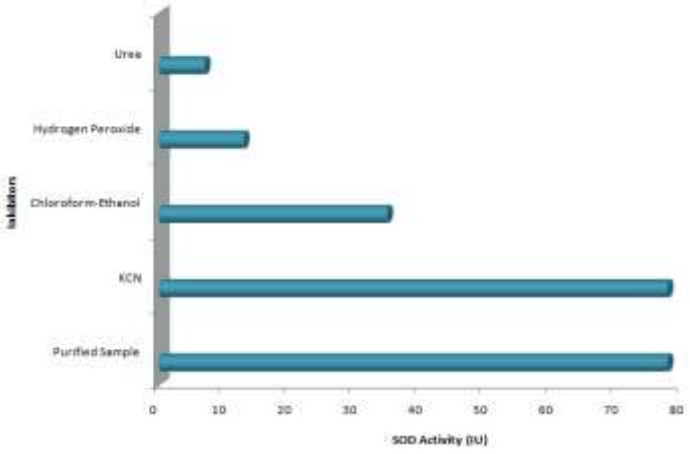

Figure 7: Effect of inhibitors on Tomato SOD activity

Metal ion analysis

The enzyme was assayed for copper, zinc, iron and manganese ions by atomic absorption spectroscopy. The purified SOD contained 1.3 ppm Fe atoms, 0.11 ppm Mn atoms, 0.03 ppm Zn atoms while the concentration of Cu atoms was below detection level (Figure 8). These results confirmed that the purified SOD was Fe-SOD.

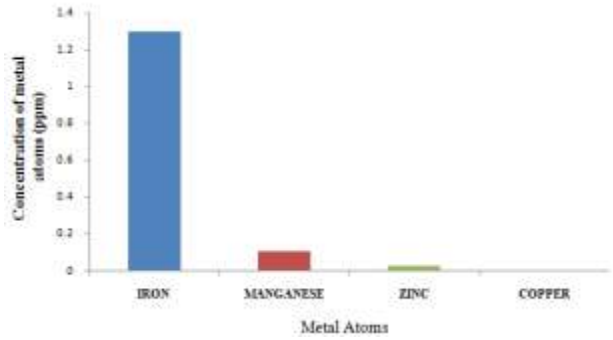

Figure 8: Metal ion analysis through atomic absorption spectroscopy

DISCUSSION

Superoxide dismutases (SOD) are a class of antioxidant enzymes that have been under considerable research for many years due to their potential use against oxidative damage caused by free radicals in the body. In the present work, isolation, purification and characterization of tomato SOD enzyme was performed and a correlation between change in total protein content or change in SOD activity with progressive ripening in tomato was established. The study started with the collection of tomato samples at nine different ripening stages and their crude extracts were analyzed for total protein content and SOD enzyme activity. A linear drop in total protein content with the progression in ripening stages was observed. This finding was similar to the already reported work [19, 20 , 21] showing that the progress in maturity of fruit may somehow be responsible for reduction in total protein content. SOD activity was also determined in all the collected samples. As per current results, SOD activity was higher in immature green stage (stage 1), that passed through a minimum level from stage 2-6, and increased again during subsequent ripening till it achieved maximum specific activity i.e. 140 IU/mg in the rotten sample (stage 9). Similar conclusion was also drawn by Jimenez et al. [19] On the contrary, SOD activity was highest at the pink stage tomato (stage 5) and declined thereafter has also been reported [22,23]. In case of Saskatoon fruit, SOD specific activity declined with ripeninig, although a peak in activity was obs erved when fruit turned to pink stage [21]. While in Hisar safeda, SOD activity increased from immature green stage to ripped stage but declined at rotten stage [24]. Mondal et al. and Reddy & Srivastava found highest SOD activity during mature green stage in tomato and mango, respectively [25, 26]. Hence, no appropriate correlation could be established between changes in SOD activity during subsequent ripening stages because of the various conflicting reported results.

The rotten tomato crude extract (stage 9) with maximum SOD activity was selected and subjected to various steps of purification (ammonium sulfate precipitation, dialysis and gel filtration chromatography). The tomato SOD was purified upto 6 fold and its molecular weight was found to be 16 kDa after SDS-PAGE analysis under denaturing conditions. This size was comparable to 15.9 kDa SOD of *Lens esculenta* and 15.1 kDa SOD of *Panax ginseng* which is a well-known medicinal herb in Asia [27]. On the contrary, purified SOD from this report had lower molecular weight from previously reported size of tomato SOD i.e. 31.5 kDa [28] and ber SOD i.e. 35.6 kDa [29].

Analysis of other properties of the purified metalloenzyme from this study disclosed that it was stable under mesophilic temperature range i.e. up to 37^{o}C and between acidic to neutral pH ranges i.e. pH 5-7. Also, it was sensitive to chemicals such as chloroform-ethanol, H_2O_2 and urea, as they reduced the activity of purified SOD (78 IU) to 35 IU, 13 IU and 7 IU respectively while KCN had no effect on its activity showing the same 78 IU as that of purified sample, suggesting that the enzyme was Fe-SOD and these observations coincided with already reported results [27, 30]. The presence of Fe-SOD was also confirmed by atomic absorption spectroscopy.

CONCLUSION

The useful information gathered from current research work could be useful in purifying and characterizing SOD enzyme from other plant sources. Also, this purified antioxidant metalloenzyme could be provided to pharmaceutical industries to be packaged in various drug forms and may be used in cosmetic products in future as well.

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