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Activity of Purified Bacterial Myrosinase and its Essential Residues

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A B S T R A C T

Myrosinase (thioglucoside glucohydrolase or thioglucosidase) (EC 3.2.3.147) hydrolyzes the glucosinolate to thiocyanates, isothiocyanates, epithionitrile, nitriles, oxazolidine-thione. Myrosinase was isolated from Bacillus thuringiensis. The enzyme was partially purified by ammonium sulfate and Sephadex G-200. The specific activity was 64 units mg-1 protein. The optimal pH and temperature of the enzyme were 8.0 and 50 °C, respectively. The optimal incubation time was 40 min. The chelating agent α-α-dipyridyl inhibited the enzyme activity in concentration-dependent manner. Adding Triton X─100 at various concentrations resulted in an increase of the enzyme activity. Diethylpyrocarbonate (DEPC), N-ethylmaleimide (NEM), N-bromosuccinimide (NBS) and phenylglyoxal (PGO) inhibited the enzyme activity at the various tested concentrations and the inhibition was dependent on the concentration of each tested compound. Mercaptoethanol activated the enzyme activity at the tested concentrations 20, 40 and 60 mM then the activity declined with increasing the concentration. GA3 as phytohormone activated myrosinase continuously up to 80 µmol then declined at 100 µmol. The thiol compounds L-methionine, N-acetylcysteine, cysteine, thiourea and dithiothreitol (DTT) activated the enzyme and N-acetylcysteine was the best activator.

Introduction

Enzymes are biocatalysts for wide number of chemical reactions. The microbial enzymes are involved in industry and medicine. The microbial enzymes are more stable than those isolated from plants and animals (Buchholz *et al.*, 2012).

Microorganisms represent the best source of enzymes because of two reasons. Firstly, they can be cultured giving large quantities of enzymes in a short time. Secondly, they have susceptibility to gene manipulation and biochemical diversity. The microbial enzymes characterized by their wide-spread with numerous industrial commercial applications. Various industries are looking for new microbial strains to produce various enzymes to fulfill the current enzyme requirements (Cavicchioli *et al.*, 2011, Anbu *et al.*, 2013).

Application of various enzymatic processes in the chemical industry depends mainly on cost competitiveness as well as established

chemical methods. Among the main advantages of the biotechnological processes compared to well-established chemical processes are lower energy demand, increased product titer, increased catalyst efficiency, less catalyst waste and byproducts, as well as lower volumes of wastewater streams (Buchholz *et al.*, 2012).

In plants, myrosinase (EC 3.2.1.147) is known to be a cytosolic enzyme; however, the cellular organization of the myrosinase–glucosinolate system remains unclear. Brassicales contain a myrosinase enzyme that hydrolyzes glucosinolates to form toxic isothiocyanates, as a defense against bacteria, fungi, insects and herbivores including man (Verkerk *et al.*, 2009). Myrosinase is located in the adjacent phloem parenchyma (Koroleva *et al.*, 2010).

The products of myrosinase action on glucosinolates are used in reducing various types of cancers in human (Dufour *et al.*, 2015), alternative biocidal compounds (Dinkova-Kostova and Kostov, 2012), create several flavors such as isothiocyanate was responsible for the hot flavors of condiments made from mustard and horseradish, and sinigrin are the cause of bitterness of some Brassica vegetables (Van Doorn *et al.*, 1998, Angelino *et al.*, 2015).

Microorganisms including bacteria and fungi have been reported for their glucosinolate-degradation. Among these microorganisms are Lactobacillus agilis strain R16 (Palop *et al.*, 1995), *Aspergillus calvatus* and *Fusarium oxysporum* (Smith *et al.*, 1993), *Aspergillus* sp. NR-4201 (Sakorn *et al.*, 1999) and *Aspergillus* sp (Rakariyatham *et al.*, 2005). Also, it has also been assayed in bacteria associated with mammal gut microflora (Fahey *et al.*, 2012).

The aim of the present work was to isolate and purify myrosinase from *Bacillus thuringiensis*. In addition, it aimed to study the biochemical characteristics of the enzyme.

Materials and Methods

Source of the Enzyme

Bacillus thuringiensis was the organism used in the isolation, purification of myrosinase and studying the response of the enzyme to various effectors.

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Growth of *Bacillus thuringiensis*

The pure culture was inoculated into a 250 ml conical flask containing 250 ml of culture medium (peptone 1g, yeast extract 0.5 g, K2HPO4 0.3g, MgSO4.7H2O 1g, sinigrin 1g, distilled water 250 ml, pH 7.0) followed by incubating at 37 °C on shaker for 24h.

Preparation of Myrosinase Extract

The culture filtrate of *Bacillus thuringiensis* grown for 72 h at 37 °C was obtained by centrifugation at 5000 rpm and used as extract of myrosinase.

Partial Purification of Myrosinase

The obtained crude extract of myrosinase was precipitated using (NH4)2SO4 with 85% saturation. It was left at 4°C overnight then centrifuged at 6000 rpm for 20 min. The pellet was dissolved in 100 mM borate buffer (pH 7.5). The resulting suspension

was examined for enzyme activity, then subjected to Sephadex G-200.

Assay of Myrosinase

Myrosinase activity was assayed according to the method of Palmieri et al. (1986). Assays was carried out using 5 mM potassium phosphate buffer (pH 7.0), containing 0.5 mM sinigrin, in a total volume of 3 ml. The assay buffer was equilibrated at 37 °C. The reaction was initiated by adding of 50 µl of enzyme solution. The decrease in the absorbance at 227 nm. The enzyme activity was expressed as 1 µmol of sinigrin per min under standard assay conditions. One unit of myrosinase activity was defined as the amount of enzyme that catalyzed the liberation of 1 µmol of glucose per min from sinigrin under the above described conditions.

Protein Determination

Protein concentration was determined spectrophotometrically according to Bradford (1976).

Optimization of pH and Temperature

The pH was optimized using pH range of 4-10. The temperature was optimized using temperature range of 10-70 °C. Optimization of the incubation time for the reaction mixture was carried out in the range 10-60 min.

Effect of Effectors and Modifying Agents

The chelating agent α-α-dipyridyl was tested at various concentrations 0.2, 0.4, 0.6, 0.8 and 1.0 mM. Gibbrellic acid (GA3) was tested at 20, 40, 60, 80 and 100 µmol TritonX-100 was tested using various concentrations 0.2, 0.4, 0.6, 0.8 and 1.0 % (v/v). Diethylpyrocarbonate (DEPC), N-bromosuccinimide (NBS) and N-ethylmaleimide (NEM) were examined at 1,2,3,4, and 5 mM in the reaction mixture. Mercaptoethanol was tested at 20, 40, 60, 80 and 100 mM. The thiol compounds L-methionine, N-acetylcysteine, cysteine, thiourea and dithiothreitol (DTT) were tested at 5mM in the reaction medium.

Results and Discussion

Myrosinase was purified by 85 % ammonium sulphate precipitation followed by Sephadex G200. The obtained specific activity was 64 units mg-1 protein (Table 1). The enzyme from cauliflower seedlings showed a lower specific activity of 12.71 units/mg protein (Prakash *et al.*, 2013).

Effect of pH on Myrosinase Activity

The activity was determined at various pH values (4, 5, 6, 7, 8, 9 and 10). The results in Fig. 1 illustrate that there was a continuous increment in the activity of myrosinase up to pH 8.0 after which the activity declined at pH 9 and 10. It appears that the optimal temperature is 8.0. However, the enzyme from Lepidium latifolium L. (Bhat *et al.*, 2015) and cauliflower seedlings (Prakash *et al.*, 2013) was 6.0. The maximum activity was observed below pH 5 for myrosinase from broccoli (Mahn *et al.*, 2014). The enzyme from horseradish root exhibited high activity at broad pH (pH 5.0–8.0) (Li and Kushad, 2005).

Effect of Temperature on Myrosinase Activity

The effect of temperature on the enzyme activity was determined at various temperatures (10, 20, 30, 40, 50, 60 and 70 °C) was investigated. The results in Fig. 2 show that at the beginning there was a continuous increase in the enzyme activity

from 10 °C to 50 °C, then the enzyme activity has reduced at 60 °C and 70 °C. Thus, the optimal temperature was 50 °C. These results are in harmony with those reported for the enzyme from Lepidium latifolium L. (Bhat *et al.*, 2015) and cauliflower seedlings (Prakash *et al.*, 2013).

Lower optimal temperature 40 °C was observed for myrosinase from broccoli (Mahn *et al.*, 2014), however the horseradish enzyme exhibited optimal temperature at range of 37-45°C (Li and Kushad, 2005).

Effect of Incubation Time on Myrosinase Activity

The effect of incubation time on enzyme activity was investigated at various time intervals (10, 20, 30, 40, 50 and 60 min). The results in Fig. 3 show that by increasing the incubation time there was an increment in myrosinase activity up to 40 min then the activity declined at 50 and 60 min.

Effect of the Chelating Agent α-α-dipyridyl on Myrosinase Activity

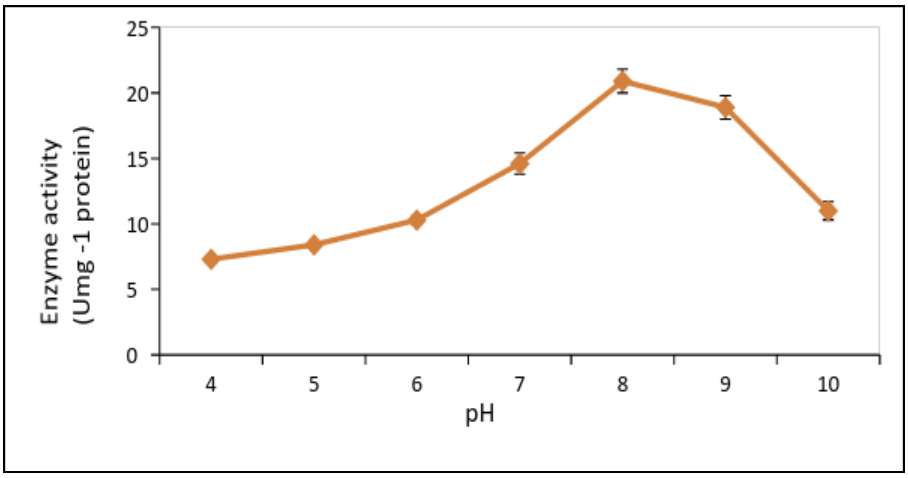
This experiment was carried out to illustrate the effect of α,α-dipyridyl as chelating agent on the enzyme activity. This compound was tested at various concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mM). The results in Fig. 4 show that increasing the concentration of α,α-dipyridyl resulted in continuous decrease of enzyme activity until it reached 1.8 units only. The inhibition of myrosinase in the present investigation by this compound reveals that this enzyme is metalloenzyme. However, myrosinase from Lepidium sativum seedlings was not inhibited by α,α-dipyridyl and phenanthroline (Durham and Poulton., 1990) for the enzyme from Lepidium sativum seedlings.

Effect of Triton X─100 on Myrosinase Activity

In this experiment the influence of Triton X─100 as a surfactant on the enzyme activity was investigated. The Triton X─100 was tested at various concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 % (v/v). The results recorded in Fig. 5 show that increasing the concentration of Triton X─100 led to a continuous increase in the enzyme activity. It was noticed that at the higher concentrations 0.8 and 1.0% (v/v) the increase in the activity was not remarkable. The activity of myrosinase was increased in the presence of Triton X-100 as non-ionic surfactant in concentration-dependent manner. The enhancement of myrosinase activity by surfactant is consistent with the results for other enzymes (Najafi *et al.*, 2005). Solubilization of enzymes by surfactants may lead to unmasking of the hidden catalytic sites by release of an allosteric inhibitory mechanism or by conformational changes in the molecule which renders the enzyme more active (Mandviwala and Khire, 2000).

Effect of Diethylpyrocarbonate (DEPC) on Myrosinase Activity

DEPC is a reagent for histidyl group in proteins (El-Shora, *et al.*, 2008). So, to test the effect of DEPC as a histidine reagent it was used at various concentrations (1, 2, 3, 4, and 5 mM) in the reaction mixture. The results in Fig. 6 show that the enzyme activity decreased continuously in a concentration-dependent manner. At 6 mM the enzyme activity was 1.6 units mg-1 protein presenting 11.7 % relative activity. The inhibition of myrosinase by DEPC indicates the necessity of histidyl group for enzyme catalysis. These results are in harmony with those reported previously by Durham and Poulton (1990) for the enzyme from Lepidium sativum seedlings.



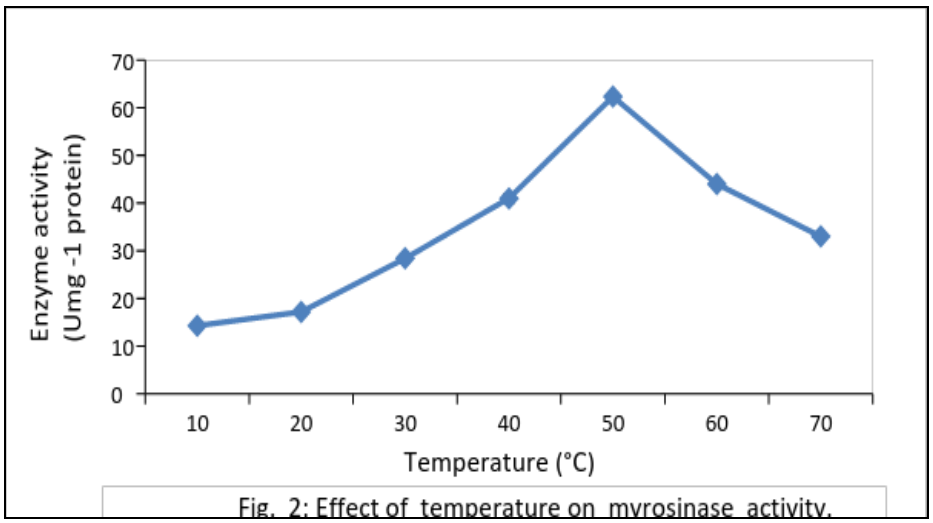


Fig. 2: Effect of temperature on myrosinase activity.

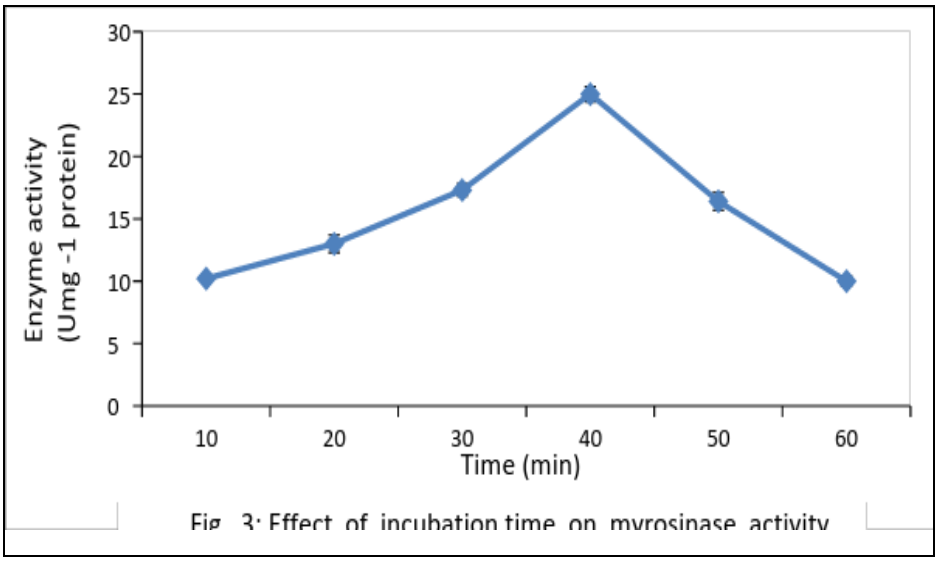


Fig. 3: Effect of incubation time on myrosinase activity

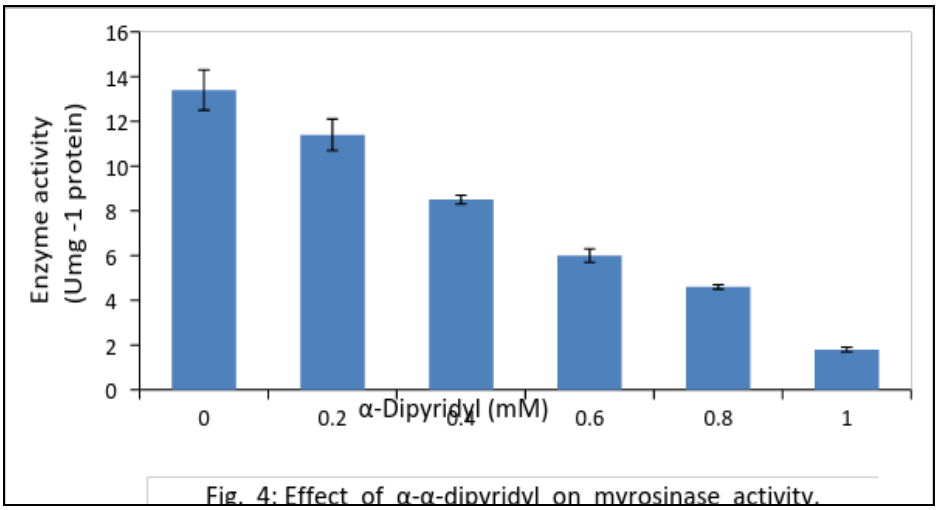


Fig. 4: Effect of α-α-dipyridyl on myrosinase activity.

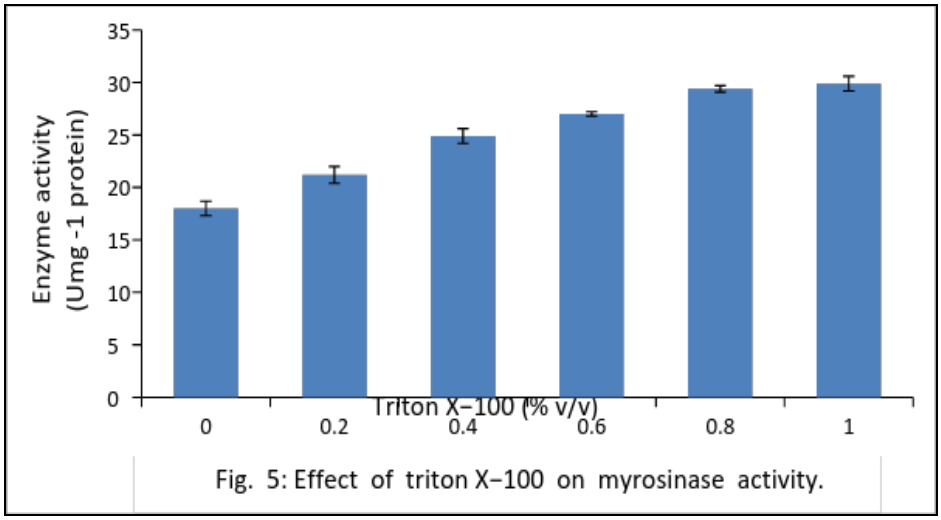


Fig. 5: Effect of triton X–100 on myrosinase activity.

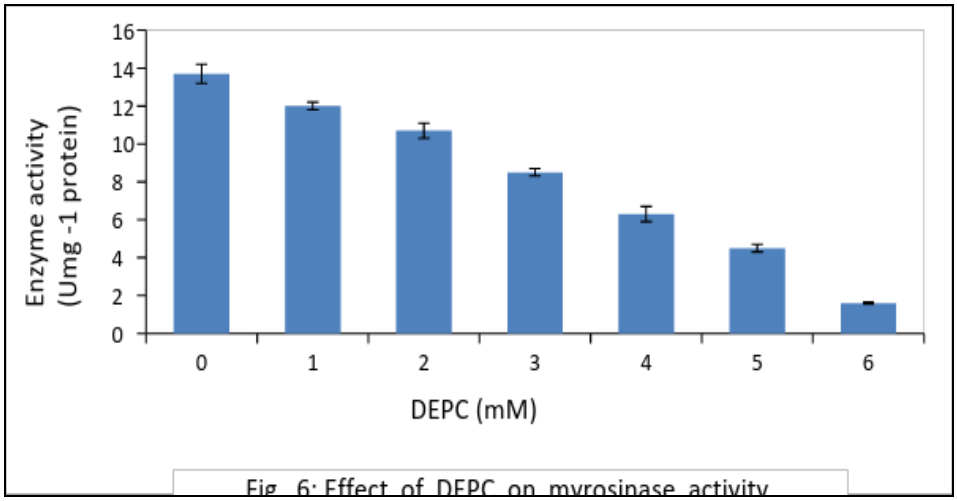
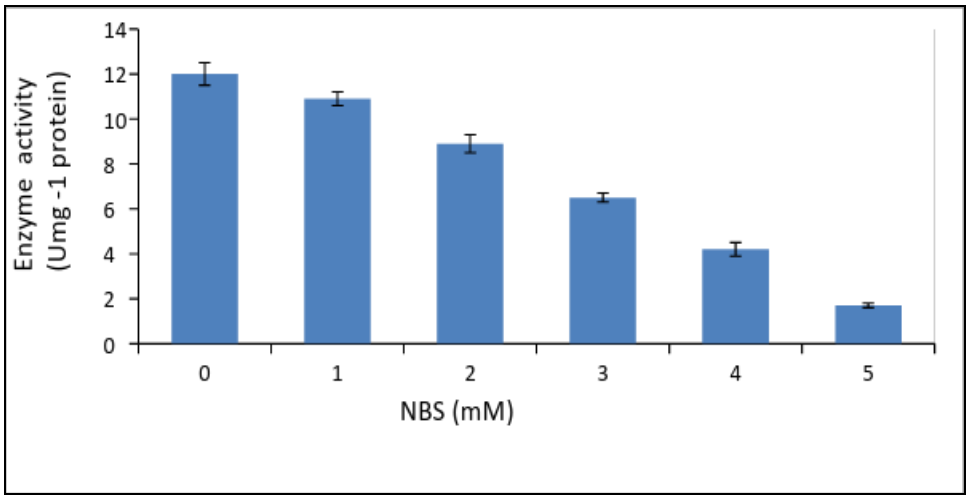


Fig. 6: Effect of DEPC on myrosinase activity



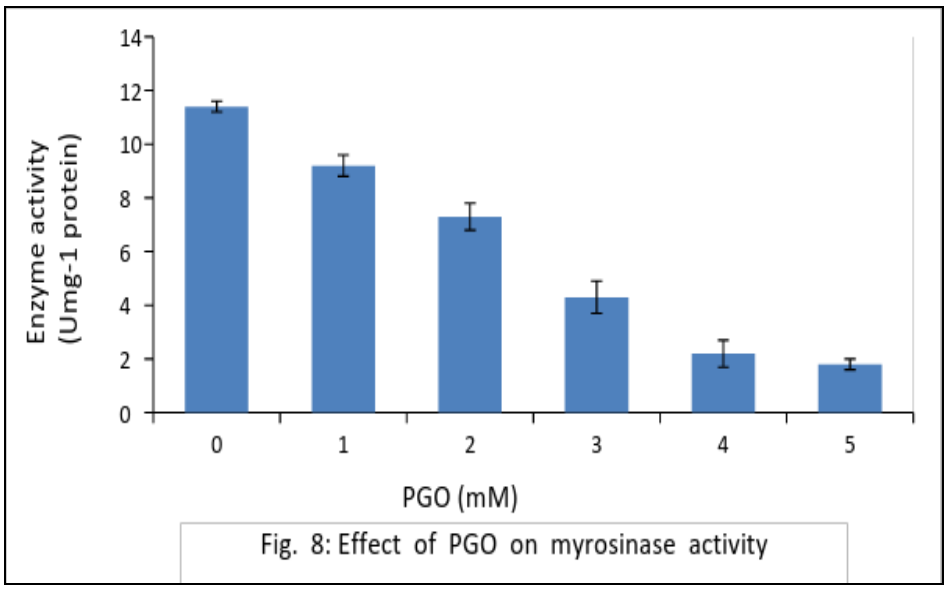


Fig. 8: Effect of PGO on myrosinase activity

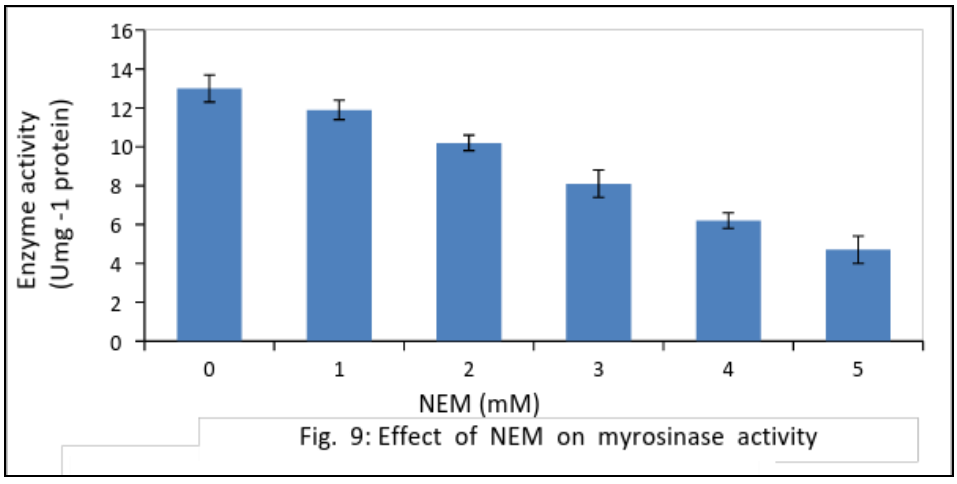


Fig. 9: Effect of NEM on myrosinase activity

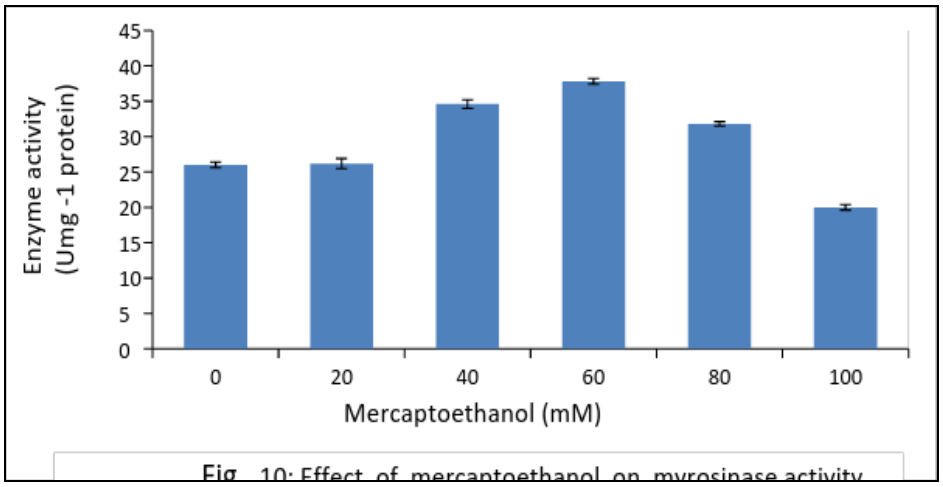


Fig. 10: Effect of mercaptoethanol on myrosinase activity

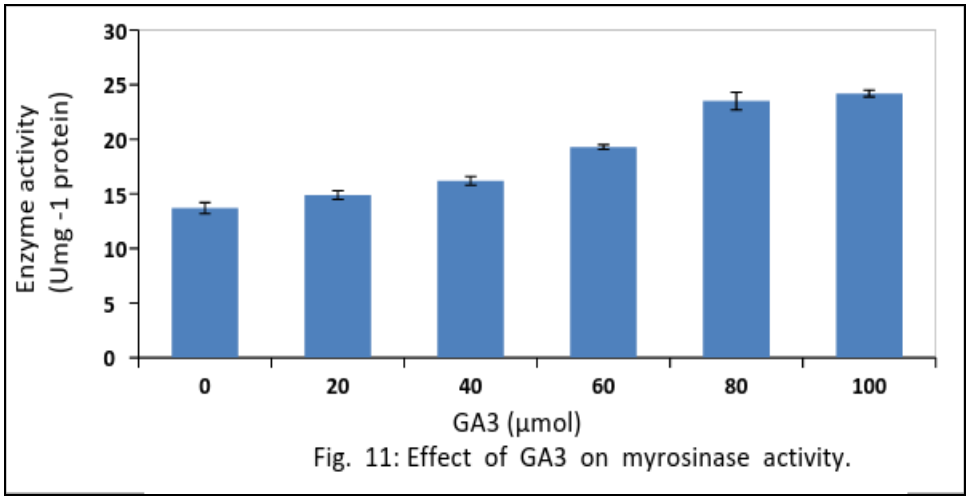


Fig. 11: Effect of GA3 on myrosinase activity.

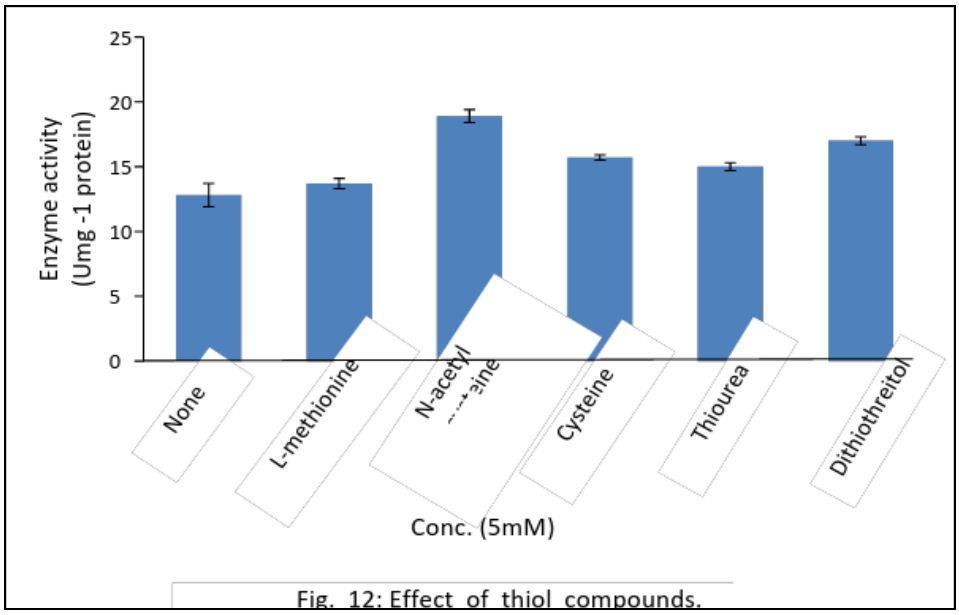


Fig. 12: Effect of thiol compounds.

Effect of N-bromosuccinimide (NBS) on Myrosinase Activity

N-bromosuccinimide is a reagent for tryptophanyl residue in enzyme protein (El-Shora *et al.,* 2009, El-Shora *et al.,* 2015). Therefore, it was decided in this experiment to test the effect of various concentrations (1, 2, 3, 4 and 5 mM) of NBS in the reaction mixture. The results in Fig. 7 show that by increasing the concentration of NBS there was a corresponding reduction in the enzyme activity until it reached 1.7 Umg-1 protein at 5 mM and the relative activity was 14.2% at this concentration. The inhibition of myrosinase by NBS is indicative for the presence of tryptophanyl and this is consistent with the results of Ohtsuru and Hata (1972).

Effect of Phenylglyoxal (PGO) on Myrosinase Activity

PGO is well known a regent for arginyl group in the enzyme protein (El-Shora *et al.,* 2015). The effect of PGO on myrosinase activity was investigated at various concentrations (1, 2, 3, 4 and 5 mM). This compound was added to the reaction mixture. The results in Fig. 8 reveal that the PGO is an inhibitor for myrosinase. The inhibition was dependent on the concentration. The relative activity at 5 mM was 1.8 units mg-1 protein with relative activity of 15.8 %. The inhibition of myrosinase by PGO proves that arginyl residue plays important role in enzyme catalysis.

Effect of N-ethylmaleimide (NEM) on Myrosinase Activity

NEM is a reagent for the sulfhyryl residues in the enzymes protein (El-Shora and Abo-Kassem, 2001). NEM on the enzyme activity was investigated. NEM is used at various concentrations (1, 2, 3, 4 and 5 mM) in the assay medium. The results in Fig. 9 indicate that the enzyme activity decreased gradually with increasing the concentration of NEM in concentration-dependent manner. The activity of the enzyme was reduced to 4.7 units mg-1 protein at 5 mM with 36.2 % relative activity. These results are consistent with those reported by Durham and Poulton (1990). These results indicate the essentiality of sulfhydryl group for enzyme catalysis.

Effect of Mercaptoethanol on Myrosinase Activity

The effect of mercaptoethanol on the activity of myrosinase was studied. Mercaptoethanol was tested at various concentrations (20, 40, 60, 80 and 100 mM). The results in Fig.10 reveal that mercaptoethanol activated myrosinase in concentration-dependent manner. It was observed that the activity of myrosinase at 100 mM was twice that recorded for the control. Mercaptoethanol as a thiol-compound enhanced other enzymes activity such as protease activity (Kamran *et al.,* 2015). The increase in enzyme activity may indicate that SH- group of the enzyme was protected by mercaptoethanol during the incubation time. Also, it is possible that these thiol compounds may lower the Km of the enzyme to its substrate and thus activating the activity.

Effect of Gibberellic Acid (GA3) on Myrosinase Activity

The effect of GA3 as phytohormone at various concentrations (2, 4, 6, 8 and 10 µmol) on myrosinase activity was studied. The results obtained are shown in Fig. 11. The results indicate that GA3 was activator for myrosinase and the activation was concentration-dependent. It was noticed that at the highest concentration (10 mM) the enzyme activity was 24.2 units mg-1 protein

which is twice that of the control value.

Activation of myrosinase by GA3 could be attributed to activation of enzyme molecules in the reaction mixture. GA3 activated other enzymes such as phosphoenolpyruvate carboxylase (Bihzad and El-Shora, 1996), sucrose phosphate synthase (Kaur *et al.*, 2000), NADH-glutamate synthase (El-Shora, 2001), acid phosphatase (El-Shora and Metwally, 2009) and cyanide hydratase (El-Shora *et al.*, 2014).

Effect of Other Thiol Compounds on Myrosinase Activity

In this experiment various thiol compounds including L-methionine, N-acetyl- cysteine, cysteine, thiourea and dithiothreitol were tested regarding their effects on myrosinase activity. These compounds were tested at 5 mM. The results in Fig. 12 show that all the tested compounds were activators for myrosinase. The best activator was N-acetyl cysteine followed by dithiothreitol which exhibited 17 units mg-1 protein. This activity represents nearly one and half of that recorded for the control. Cysteine activated other enzymes such as asparaginase (Warangkar and Khobragade, 2010). Thiourea activated other enzymes such as asparaginase (Warangkar and Khobragade, 2010). Dithiothreitol protects notably enzyme activity loss which occurs by the oxidation of sulfhydryl groups (Alliegro, 2000; El-Shora, 2001; El-Shora and Metwally, 2009). This may indicate that SH- group of the enzyme was protected during the incubation time. Also, it is possible that these thiol compounds may lower the Km of the enzyme to its substrate and thus activating the activity.

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