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Modifications of cresolase activity of *Crataegus Spp* in presence of Detergents, Inhibitors, Ionic detergents and Metal Ions

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Abstract

Polyphenol oxidases are enzymes that catalyze the oxidation of phenolic compounds using molecular oxygen. Cresolase activity of *Crataegus SPP* decreased with increasing detergents, chaotropic agents, inhibitors and metal ions concentration. The most effective concentration for inhibition of cresolase by sarcosyl, sodium cholate, Tx-100, NP-40, urea and GnHcl were 1 mM, 2 mM, 5 µM, 5 µM, 100 mM and 100 mM, where the measured activity was 28%, 69%, 96%, 98%, 86% and 81% of control, respectively. The activity increased 25% in presence of 0.5mM of sarckosyl and was also decreased at 1mM, 0.28-fold by exposure to sarkosyl, 0.69- fold by exposure to sodium cholate, 0.96- fold by exposure to Tx-100, 0.98- fold by exposure to NP-40, 0.86- fold by exposure to Urea and 0.81- fold by exposure to GnHcl. Therefore detergents and chaotropic agents are inhibitors of cresolase in Crataegus Spp that can probably change conformation of enzyme. Inhibitory effect of Urea, GnHcl, NP-40, Triton x-100 and Sodium cholate in presence of these substrates investigated so urea, GnHcl, Tx-100 and NP40 showed that they are potent inhibitor of cresolase and sarkosyl showed that it is a weak inhibitor of cresolase. The enzyme was totally inhibited in the presence of ascorbic acid, citric acid, sodium metabisulphate and benzoic acid and highly activated with manganez ions whereas other agents or metal ions such as Cu2+, Zn2+, Ni2+, Al3+, Cd2+ and Cr3+ showed inhibitory effect on activity. Cresolase activities inhibited by addition of citric acid, sodium metabisulphite, ascorbic acid and benzoic acid. Mn^{2+} and Co^{2+} increased activities by 3.69 and 1.48-fold, respectively.

Keywords: P-cresol, Tx-100, Purification, Mn^{2+} **,** Co^{2+} **.**

1. Introduction

Polyphenol oxidases (PPOs) are enzymes, belonging to a group of copper-containing metalloproteins and are members of oxidoreductases, that catalyze the oxidation of a wide range of phenolic compounds by utilizing molecular oxygen [1, 2]. There are mainly three types of polyphenol oxidases classified according to their substrate specificities and mechanism of actions. These are; tyrosinase, cresolase and laccas. Many vegetables and fruits become discoloured during storage or processing, an action mediated by the enzyme polyphenol oxidase (PPO) [3]. PPO (tyrosinase, EC 1.14.18.1) is a copper-containing enzyme that is widespread in plants, and synthesised early in tissue development and stored in chloroplasts [4]. The enzyme is a copper protein widely distributed in a multitude of organisms, from bacteria to mammals [5]. Enzymatic browning is the main function of PPOs in fruits and vegetables but is often undesirable and responsible for unpleasant sensory qualities as well as losses in nutrient quality [6]. These highly reactive quinones polymerize with other quinones, amino acids and proteins to produce coloured compounds, and nutrient quality and attractiveness is reduced [7]. PPO from different plant tissues shows different substrate specificities and degrees of inhibition. Therefore, characterisation of the enzyme could enable the development of more effective methods for controlling browning in plants and plant products. Our objective was to characterise PPO from hawthorn cultivated in Kurdistan, Iran under different conditions. Substrate and temperature effects were also studied.

2.Experimental

2.1 Chemicals**:** The Hawthorn used in this study were obtained from Kurdistan of Iran and frozen at -25 °C until used.

polyvinylpyrolidone (PVPP), sodium cholate were purchased from Merck (Darmstadt,Germany).Phenylmethylsulfonyl fluoride (PMSF), nonaaethylene glycol octylphenol ether (Triton x-100), nonaethylene glycol octylphenyl ether (NP-40), urea and guanidine chloride (GnHcl) were purchased from Sigma-Aldrich (St. Louis, USA). Sarkosyl was purchased from Fluka; All chemicals were of analytical grade.

2.2 Enzyme Extraction : 650 grams of Hawthorn were homogenized in 300 mL of 0.1M phosphate buffer (pH 6.7) containing 10 mM ascorbic acid and 0.5% polyvinyl pyrrolidone with the aid of a magnetic stirrer for 1h. The crude extract samples were centrifuged at 30000 g for 20 min at 4ºC. Solid ammonium sulphate (NH4)2SO4 was added to the supernatant to obtain 30 and 80% (NH4)2SO4 saturation, respectively. After 1 h, the precipitated proteins for each stage were separated by centrifugation at 30000 g for 30 min. The precipitate was redissolved in a small volume of distilled water and dialyzed at 4ºC against distilled water for 24 h with 4 changes of the water during dialysis.

2.3 Protein Determination : Protein contents of the enzyme extracts were determined according to lowry method using bovine serum albumin as a standard [8].

2.4 Enzymatic activity assays**:** Assays were conducted at room temperature (~22-25°C), unless otherwise stated. Enzymatic activity was determined by measuring the increase in absorbance at 400 nm for p-cresol with a spectrophotometer (6305 JENWAY). In order to correct for substrate autoxidation, the reaction mixture, was placed in the sample cuvette while the reference cuvette contained buffer and the substrate. Enzyme activity was calculated from the linear portion of the curve. Appropriate aliquots of 5 mM detergents and agents prepared in 0.1 M phosphate buffer, pH 6.7, were added to the reaction mixture just before addition of the Hawthorn extract. The final volume of the reaction mixture was always 3 ml.

2.5 Effect of Detergents and Chaotropic Agents: The enzymatic activity were also measured in the presence of increasing concentrations of ionic detergents (Sodium cholate and sarkosyl), Nonionic detergents(Triton x-100 and NP-40) and Chaotropic agents (Urea and GnHcl). The concentrations used in each case were kept within the range where no aggregation was observed and were determined as a function of the sensitivity of the enzymatic activity. Enzymatic activity of Cresolase assayed in extract and in partially purified Cresolase separately.

2.6 Activity Assayes in the presence of Ionic Detergents: The Cresolase activity was assayed in 0.1 M phosphate buffer at pH 6.7 with 15 mM p-cresol as the substrate in the presence of different concentrations of sarkosyl and sodium cholate. The concentration ranges remained below the critical micelle concentration (CMC) for each detergents.

2.7. Activity Assays in the presence of Non Ionic Detergents: The Cresolase activity was assayed in 0.1 M phosphate buffer at pH 6.7 with 15 mM p-cresol as the substrate, in the presence of different concentrations of Triton x-100 and Np-40. The concentration ranges remained below the critical micelle concentration(CMC) for each detergents.

2.8. Activity Assayes in the presence of chaotropic Agents: The Cresolase activity was assayed in 0.01 M phosphate buffer at pH 6.7 with 15 mM p-cresol as the substrate in the presence of different concentrations of urea or GnHcl. Cresolase activity assayed in extract and in partially purified Cresolase separately.

2.9. Effect of general cresolase inhibitors on partial purified activity

The following compounds were evaluated for their effectiveness as an inhibitor of cresolase activity using p-cresol as substrate: benzoic acid, sodium metabisulphite, Citric acid and ascorbic acid. An aliquot of each inhibitor at various final concentrations was added to the standard reaction solution immediately before the addition of 75 µl enzyme extract. Relative enzymatic activity was calculated as a

percentage of the activity in the absence of inhibitor. The concentration of inhibitor giving 50% inhibition (I50) was determined from plot of residual activity against inhibitor concentration.

2.10. Effect of metal ions on cresolase activity

The effects of ten metal ions were evaluated on cresolase–PPO activity, using 3ml reaction mixture (75 µl enzyme, p-cresol 15 mM at phospahate buffer. The change in absorbance was measured spectrophotometrically at 400 nm. Co2+, Ca2+, K+, Mn2+, Cu2+, Zn2+, Ni2+, Al3+, Cd2+, and Cr3+ were used to study the effect of metal ions on cresolase activity. After addition of each metal ion solution at 1 mM final concentration, the activity was assayed using p-cresol as substrate. The percentage remaining activities were expressed by comparison with standard assay mixture with no metal ion added. Thus the effects of various inhibitors on enzyme activity were examined by incubating a mixture consisting of 75 µL of the enzyme solution and 50 µL of the inhibitors (1mM) for 5 min at room temperature. Then enzyme activity was assayed under standard conditions. Enzymatic activities were expressed as relative values (percent) with reference to the activity of the enzyme without any of these respective reagents.

3. Results and discussion

3.1. Assays conduct with Hawthornes crude extract at pH 6.7: Cresolase activity in presence of p-cresol assayed as described in materials and methods, were detectable in Hawthorn extract. Under our experimental conditions, no lag period was observed in the expression of either activity.

3.2. Effect of Ionic detergents. *Effect of sarcosyl* : The addition up to 0.5 mM sarkosyl to the reaction mixture resulted up to a 25% increase in the activity when p-cresol was used as the substrate at pH 6.7 . Increasing of sarkosyl up to 1 mM showed inhibitory effect, so 28% decrease in activity was obtained(Figure 1). Over the concentration range (0-1mM) , sarkosyl had inhibitory effect on Cresolase activity in presence of p-cresol, although higher concentrations of sarkosyl, cloudiness prevented further investigations with these substrate (table 1).

3.3. Effect of sodium cholate: In the presence of sodium cholate at pH 6.7, a decrease in Cresolase activity was observed when p-cresol was used as the substrate. The inhibition increased with increasing detergent concentration, until a plateau was reached. In the presence of sodium cholate(0.5 mM), the Cresolase activity decreased progressively to 38 % of the control. The detergent concentration increased progressively to 2 mM in presence of p-cresol. Cresolase activity reached to 31%, so, showed 69% Inhibition of Cresolase. (Figure 2).

3.4. Effect of Nonionic detergents at pH 6.7: The addition of Triton x-100 or NP-40 to the reaction mixture at pH 6.7 led to a decrease in the Cresolase activity, whether p-cresol was used as the substrates. The extent of inhibition, however varied with the detergent used. With p-cresol, up to 68% inhibition was observed in the presence of 2 µM of triton x-100. 74% inhibition was observed in the presence of 2 µM NP-40 (Figure 3). Over the same ranges of concentrations (5 µM for triton x-100 and NP-40), activity of Cresolase decreased to 96 and 98% of control.

3.5. Effect of Chaotropic Agents: The addition of increasing of urea to the assay mixture at pH 6.7 resulted in increasing inhibition of the Cresolase activity, so that 49% inhibition was reached with 10 mM urea and increasing of urea concentration up to 120 mM caused 86% inhibition in Cresolase activity in presence of p-cresol; no further inhibition was observed for up to 300 mM urea (Figure 4).

3.6. Effect of GnHcl: Up to 36% and 81% inhibition of Cresolase activity was observed for p-cresol oxidation in presence of 10 and 120 mM GnHcl, respectively. No further inhibition was observed for up to 300 mM urea (Figure 4).

3.7. Effect of inhibitors on cresolase activity

The behaviour of hawthorn cresolases for some general cresolase inhibitors was examined. Benzoic acid (0.1–5 mM), Citric acid (0.1–5

mM), sodium metabisulphite (0.1–1 mM) and ascorbic acid (0.1–2 mM) were used as inhibitors. All the compounds used in this study inhibited the enzyme. Their potentials for the inhibition of hawthorn cresolase activities are presented as I50 values calculated from the plots of inhibitor concentrations VS. percentage inhibition of p-cresol oxidation (Table 2). Hawthorn cresolase activities were fully inhibited by addition of sodium metabisulphite, ascorbic acid and benzoic acid. Inhibition assays indicate that thiol compounds, such as metabisulphite with low I50 values, are potent inhibitors of the hawthorn cresolases, consistent with the earlier reports about plant PPOs [9,10,11]. Results showed that I50 of sodium metabisulphate < citric acid < ascorbic acid< benzoic acid.

Table2 : I50 for inhibition of cresolase activity of Crataegus SPP by some inhibitors.

Inhibition of **P-cresolase** activity

Inhibitor	% Inhibition
Ascorbic acid	64 ± 5
Citric acid	37 ± 4
Benzoic acid	76 ± 3
Sodium metabisulphite	24 ± 2

3.8. Effect of metal ions on cresolase activity

A wide variety of proteins and enzymes incorporate metal ions or metal complexes into their overall structure, and trigger enhancement of their activity. Effects of metal ions and other chemical reagents on the enzyme activity were studied at their final concentration (1mM) using p-cresol as the substrate. As shown in table2, addition of Co2+, Mn2+ (1mM) to the assay reaction mixture showed enhancement in PPO activity, and Cu2+, Cr3+, Zn2+, Cu2+, Al3+, Cd2+ and Ni2+ (1mM) inhibited the enzyme activity while KCl (K+) showed no effect on cresolase activity of PPO. It has also been reported that Co2+ and Mn2+ are activators of cresolase while other are inhibitors of enzyme. Since metal ions may have different coordination numbers, geometry in their coordination compounds, and potentials as Lewis acids, they may behave differently towards proteins as ligands. These differences may also result in metal binding to different sites, and therefore, perturb the enzyme structure in different ways [12,13,14]. It could be speculated that Mn(II) may activate cresolase by binding to either an allosteric or a metal binding site on the enzyme structure. How Mn(II) interact with cresolase and its stimulation of enzyme activity needs further investigation. K+ showed no effect on cresolase activity of hawthorn.

Table1 : Effect of Detergents and Chaotropic agents on Cresolase activity of Crataegus Spp in extract at pH 6.7

Detegents		***Concentartion***	***Effect on P-cresol oxidation***
Ionicd etergent	***Sarcosyl***	***0-0.5***	***25 % activation***
		0.5-1	***28 % Inhibition***
	Sodium cholate	***0-0.5***	***38 % Inhibition***
		0.5-2	***69 % Inhibition***
Non ionic detergent	***Tx-100***	***0-2 µM***	***68 % Inhibition***
		2-5 µM	***96 % Inhibition***
	NP-40	***0-2 µM***	***74 % Inhibition***
		2-5µM	***98 % Inhibition***
Chaotropic detergent	***Urea***	***0-10 mM***	***49 % Inhibition***
		10-120 mM	***86 % Inhibition***
	GnHcl	***0-10 mM***	***36 % Inhibition***
		10-120 mM	***81% Inhibition***

20 mM. The reaction medium at 25 °C contained 0.1*M* sodium phosphate buffer, pH 6.7 and increasing concentrations of sarkosyl (0 to 1 m*M*).

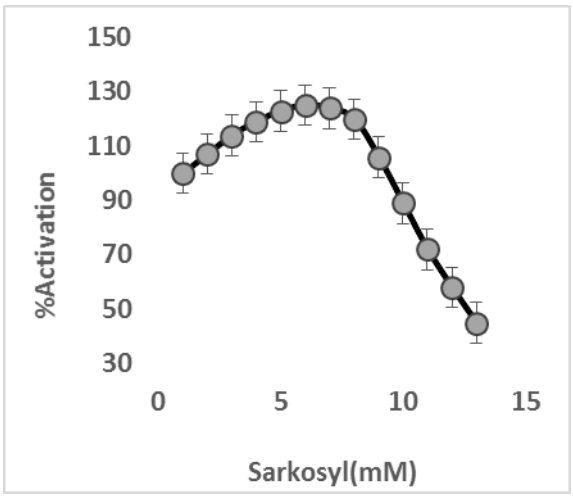


Figure 1. Effect of sarkosyl concentrations on Hawthorn cresolase activity in presence of p-cresol

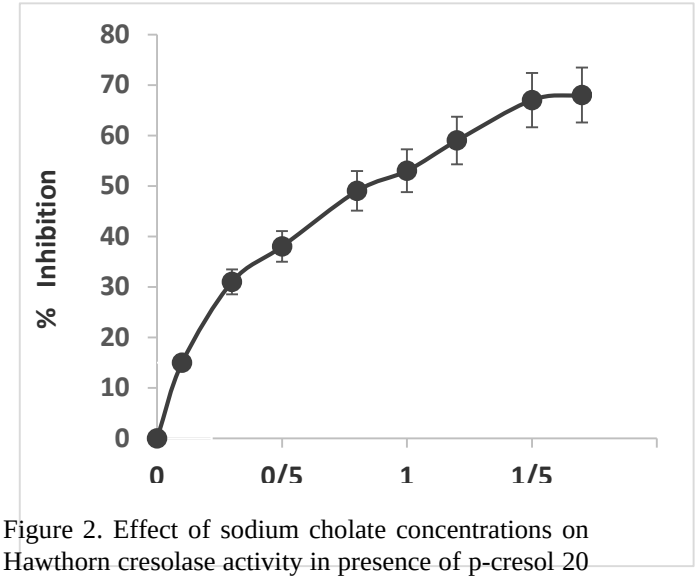
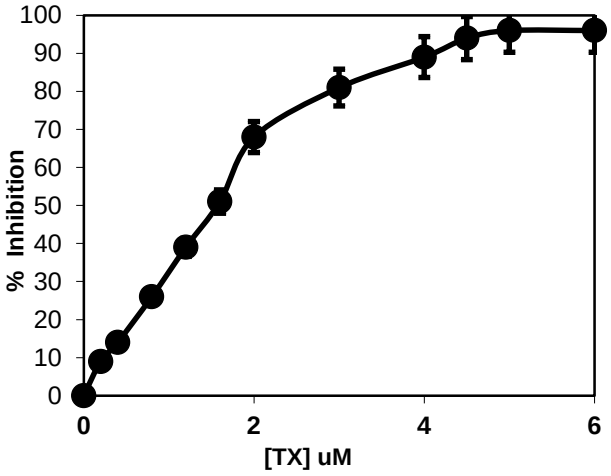


Figure 2. Effect of sodium cholate concentrations on Hawthorn cresolase activity in presence of p-cresol 20 mM. The reaction medium at 25 °C contained 0.1*M* sodium phosphate buffer, pH 6.7 and increasing concentrations of sodium cholate (0 to 2 m*M*).

Table3 . Effect of various metal ions on hawthorn cresolase activity.

Metal ion (1 mM)	***Relative activity (%)***		***Metal ion (1 mM)***	***Relative activity (%)***
None	100 ± 3		Co2+	148 ± 3
K+	98 ± 3		Mn2+	369 ± 10
Cd2+	45 ± 2		Ni2+	22 ± 1
Al3+	33 ± 2		Zn2+	34 ± 2
Cr3+	49 ± 2		Cu2+	36 ± 2



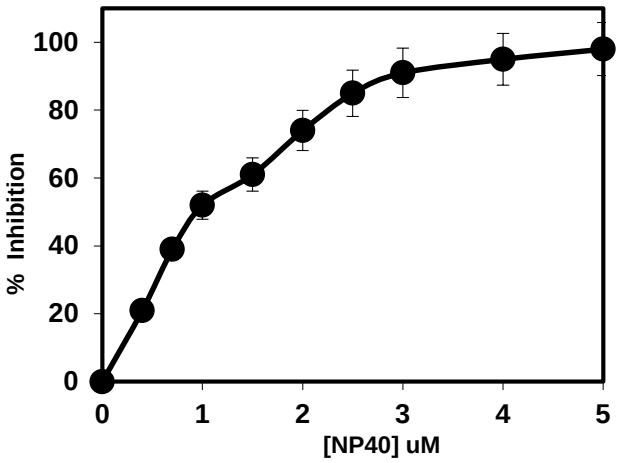
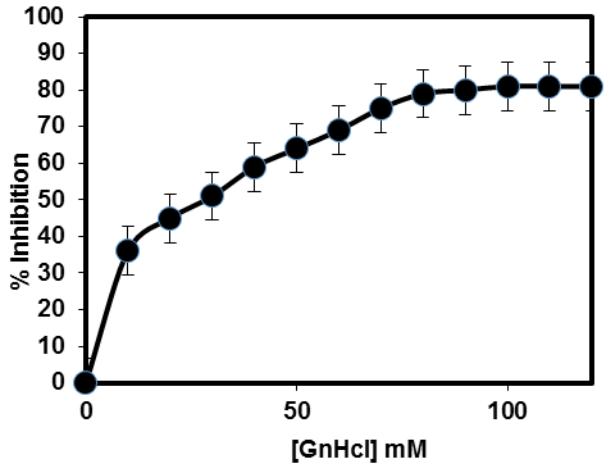


Figure 3. Effect of NP-40 and Triton x-100 concentrations on partial purified cresolase activity of Hawthorn in presence of pyrogallol 15 mM. The reaction medium at 25 °C contained 0.1*M* sodium phosphate buffer, pH 6.8 and increasing concentrations of Triton x-100 and NP-40 (0 to 5 µ*M*).



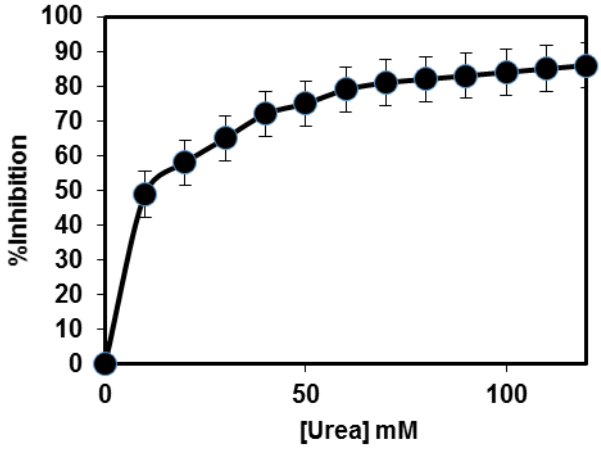


Figure 4. Effect of guanidine hydro chloride (Gn.Hcl) and Urea concentrations on Hawthorn cresolase activity in presence of pyrogallol 15 mM. The reaction medium at 25 °C contained 0.1*M* sodium phosphate buffer, pH 6.8 and increasing concentrations of Gn.Hcl and urea (0 to 120 m*M*).

4. Conclusion

The present investigation was conducted on extracts obtained from Hawthorn. The extract prepared as described in the Materials and Methods consisted of the soluble fraction of the fruit homogenate and the Cresolase activity reported here refer to the soluble enzyme. Each assay was performed at least in triplicate and each experiment was repeated three times. The results were expressed as means of the values obtained and the standard deviations of the means were calculated and shown on the figures. Cresolase retains its intriguing property of being able to exist in a latent form which it can be released by a variety of treatments, such as exposure to activators that are reputed to inactive enzymes [14]. Sarkosyl would inhibit Hawthorn Cresolase activity for oxidation p-cresol exhibited different sensitivities toward the various detergents and agents used. Cresolase activity for oxidation of p-cresol was sensitive to detergent/agent concentrations the same as activity of Cresolase for pyrogallol and p-cresol oxidation[15]. Our results showed that among the detergents/agents tested in this study sarkosyl would inhibit

Cresolase the same as other detergents that inhibited the enzyme activity. The inhibition observed with sodium cholate may be due to steric hindrance upon binding of this bulkier detergent to the enzyme. Nonionic detergents were larger than sodium cholate and may cause inhibition upon binding to the enzyme by preventing access of the substrate to the active site because of steric hindrance [16]. The present study shows that Cresolase in Crataegus SPPare inhibited by sarkosyl and by urea and other detergents. Interestingly, while no more than 86% inhibition was observed in the presence of up to 200 mM urea for oxidation of p-cresol. Similarly, GnHcl caused no more than 81% inhibition of Cresolase for oxidation of p-cresol. Thus activity of soluble Cresolase of Crataegus Spp was sensitive to chaotropic agents and nonionic detergents for oxidation of these substrates. These finding indicated probably a difference in the conformational and in the microenvironment of the respective active site in the enzyme. Hawthorn cresolase activities were fully inhibited by addition of sodium metabisulphite, ascorbic acid, citric acid and benzoic acid. Inhibition assays indicate that thiol compounds, such as metabisulphite with low I50 values, are potent inhibitors of the hawthorn cresolases. Results showed that I50 of sodium metabisulphate < citric acid < ascorbic acid< benzoic acid, so sodium metabisulphate is a potent inhibitor of cresolase activity of hawthorn. The cresolase activities increased 3.69-fold for hawthorn cresolase, in the presence of 1 mM Mn2+. Since metal ions may have different coordination numbers, geometry in their coordination compounds, and potentials as Lewis acids, they may behave differently towards proteins as ligands. It could be speculated that Mn(II) may activate cresolase by binding to either an allosteric or a metal binding site on the enzyme structure. How Mn(II) interact with cresolase and its stimulation of enzyme activity needs further investigation. K+ showed no effect on cresolase activity of hawthorn.

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