Research Article

Biochemical studies on the characters of polyphenol oxidase from different cultivar of wheat

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Abstract

Polyphenol oxidase (PPO), is well-known to induce browning in wheat-based products. In the present study biochemical study on characteristics of PPO was done and it was observed that PPO from 10 different Pakistani wheat (*Triticum aestivum* L) cultivars shows higher affinity towards L-DOPA, pH and temperature. It was found that pH 5 shows maximum activity and 30°C was optimum temperature. At various temperature ranges, study was done. The enzyme was found to be stable at different temperatures up to 70°C. Among various PPO inhibitors when tested, the most effective inhibitors for the enzyme with 10mM L-DOPA as substrate were EDTA and ascorbic acid. The weakest inhibitors were DDT *p*-coumeric acid, Ferulic acid, *Trans*-cinnamic acid, Vanillic acid and Diethyldithiocarbonate. While Cysteine was the least potent inhibitor. Sodium Chloride and Sodium bromide isoproterenol and sodium iodide *O*-phenanthroline was proved to be effective inhibitors. The effect of SDS on wheat PPOs was studied, and it was studied that PPO activity was increased in presence of 50 mM SDS. The extraction of PPOs activity was increased by adding to the extraction buffer following alcohols methanol acetone, 2-propanol ethanol, and *n*-butanol. The alcohol *n*-butanol was found to be most potent alcoholic activator, 2-propanol was next to *n*-butanol, while the least effect was observed for ethanol and methanol.

Keywords: *Triticum aestivum*; Polyphenol oxidases(PPOs); Enzyme activation; Detergents; Inhibitors

Introduction

Polyphenol oxidases (PPOs) are metallo enzymes that contain copper and after production in the nucleus, PPO moves to plastid [1]. Phenolic substrates are converted to dark-coloured polyphenols (Melanin) by the action of PPO. Brown colour of chapattis and flat breads of Middle East, noodles and other end products of wheat, are considered due to PPO [2, 3].

Among globally cultivated cereal crops, Wheat is considered to be the most important crop. Ten thousand years ago wheat was first crop cultivated. Worldwide, 17% of the cultivated area is covered by wheat. Other cereals like Maize and Rice which are grown in humid environment while wheat crop prefers temperate region. Wheat crop is the vital food for 40% population of the world [4, 5]. Wheat is grown in all parts of the world as

compared to other cereals, as it covers more of the earth surface [6]. In 94 developing countries, Wheat is on second position after rice. The total food calories provided by it is 21%. In Pakistan wheat is used in different forms like bread, breakfast cereal, Chapatti, and other various items of bakery. In East Asian nations wheat is consumed in the form of Noodles. Brown coloration is developed during storage in noodles, flour and other end products, while bright and creamy white colour is preferred by consumers [7].

In world wheat trade, the value of wheat products became a main mark in program of wheat breeding. Various physical and chemical aspects refers to the quality of wheat product as it depends on the planned purpose. The wheat grain quality is effected by different factors which are mostly categorised in to two groups, i.e chemical and physical features. In plant physiology and food science, PPO has taken much attention due to PPO adverse browning effect. Characteristics of PPO found in plant has been extensively studied in numerous vegetables, fruits, and many crops due to its importance of browning reaction in food technology and post-harvest physiology. Browning reaction can be controlled by the use of different methods. These methods include use of different substrates or extensive- range of compounds for inhibition of the enzyme, or by elimination of one of its essential constituents like, copper, enzyme and oxygen [8].

The target of present research is biochemical characterization of a wheat PPO extracted from different wheat cultivars of Pakistan, comprising affinity for PPO substrates that are used commonly and how the inhibitors effect the activity, optimum pH, temperature and stability of pH was studied. Other aims of the study is influence of the detergent SDS on PPO activity were also observed along with investigation of extract from wheat PPO with MOPS buffer that may possibly be

further stimulated by addition to the extract some known amounts of organic solvent like alcohol and ketone.

Materials and methods

Plant material

Grains of 10 different wheat cultivars (PIRSABAK 2005, SHAFaq-06, KARWAN-2, KOHINOOR 83, KOHISTAN 97, NAEEM 82, PARWAZ 94, PASBAN 90, MIRAJ-08, SEHER-06) were obtained from Nuclear Institute of Food and Agriculture (NIFA) Tarnab KPK for their polyphenol oxidase characterization,

Extraction of PPO

Extraction of PPO was done from whole grains of wheat using standardized method [9] [10]. Wheat grains were ground in liquid nitrogen and it was incubated in 1 ml of extraction buffer (50 mM MOPS pH 6.5, 0.2% SDS, 0.2 mM Pefabloc 0.2% NP-40), taken in a micro centrifuge tube and shake at (8 rpm), temperature was 4°C for 1 hour. After homogenization for 60 sec the suspension was incubated again for 1 hr in the shaker. The decanted supernatant was stored at -80 °C after suspension was centrifuged at 4 °C and 20,000 rpm for 30 minutes.

PPO activity assay with different substrates

By following the same method for each cultivar the experiment was performed in triplicate. Five grains from each cultivar were kept in test tubes comprising 1.5 mL of phenolic substrate (Hydroquinone, Resorcinol, Phloroglucinol, *p*-coumeric acid, Ferulic acid, Vanillic acid, L-Tyrosine, Pyrocatechol, 4-Methylcatechol, L-DOPA, Caffeic acid, Chlorogenic acid, (+) Catechin, (-) Epicatechin, phenol) made up in 50 mM 3-(*N*-morpholino).

For phenol, incubation of tubes was done at 20°C and was rotated at 160 rpm for 2 hours. The tubes were incubated at 25 °C rpm for 2 hours. All the other substrates were adjusted at volume of 10 mM with the exemption of phenol, which was diluted at 2 g/lit solution.

Succeeding to incubation, solutions part was removed from the tubes, change in absorbance was noted and compared with a control that was comprising only the particular substrate. Absorbance was recorded at 410 nm by spectrophotometer for catechol and phenol reactions mixtures, whereas for L-DOPA and caffeic acid reactions, absorbance changes were recorded at 475 nm.

Determination of the optimal activity

1. Effect of pH on PPO activities and stabilities

The effect of pH on PPO enzymatic activity was studied, ranging from pH 4.0 to 9.0. By incubating the compound in 0.05 M phosphate (pH 4.0 to 9.0) for 30 min, the pH stability was studied.

2. Effect of temperature on PPO activity

For finding the ideal temperature estimations of the enzyme, PPO action was measured at various temperatures ranges of 10-60 °C, utilizing five substrates of phenol comprising 3, 4-dihydroxycinnamic acid (caffeic acid), phenol 3, 4-dihydroxybenzene (catechol), L-DOPA and L-tyrosine. By heating the

standard reaction solutions (buffer and substrate) upto proper temperature before adding of the enzyme, thus impact of temperature on the action of PPO was noted.

3. Thermal stability of PPO

The thermal denaturation of the purified enzyme was learned at 35°C, 55°C, and 75 °C. One mL of standard reaction solution at temperature that is required in a test tube, was incubated for settled time intervals. Required time interval was reached, the test tube was kept in an ice bath to cool down. At 25 °C the activity of the enzyme was measured.

4. Effect of inhibitors on PPO activity

For the study of PPO inhibitors on PPO activity in each sample, 2 mL micro centrifuge tube was taken and 5 grains were placed in it and pre incubated for thirty minutes in 1.50 mL with a given inhibitor (Table 1) in 50 mM MOPS, at pH of 6.5, with 0.02 percent Tween- 20 (volume/volume) and kept at room temperature of 25 °C with continuous mixing. Following pre incubation, 0.25mL of 10 mM L-DOPA was added. The PPO activity was measured.

Table 1. Substrate specificity of PPO in wheat

Substrate	Relative activity (%)
Hydroquinone	0
Resorcinol	0
Phloroglucinol	0
<i>p</i> -coumeric acid	0
Ferulic acid	0
Vanillic acid	0
L-Tyrosine	25.0
Pyrocaticol	22.6
4-Methylcatechol	15
L-DOPA	100
Caffeic acid	5.0
Chlorogenic acid	4.5
(+)Catechin	10.7
(-)Epicatechin	30.8

PPO assays for the evaluation of SDS impact on PPO extraction and activity

For the assessment of SDS influence on PPO action and extraction, PPO from wheat cultivar was separated with 500 L medium of extraction comprising of MOPS buffer 50 mM. with 6.5 pH, and 0–100 mM SDS. The PPO activity was measured.

PPO assays for the evaluation of organic solvent impact on activity of PPO and its extraction

Wheat grain PPO was extracted with Phosphate buffer comprising of chemical compounds to study the effect of these additives. Arrangements of different concentration of the organic solvents were set up in the MOPS buffer (50 mM) at 6.5 pH. Buffer of 50 mM MOPS containing 1-40 percent (Volume/Volume) of methanol, ethanol, 2-propanol and n-butanol, were likewise arranged. These chemicals were then separately utilized for the extraction of PPO to study their impacts on the enzymatic activity. The PPO action was measured.

Results and discussion

Colour is an imperative component characteristic of products made up of wheat and polyphenol oxidase (PPO) is thought to be responsible and have noteworthy part in their detrimental browning reactions. The enzymatic reaction regularly happens in

wheat end product and is the reason for an abatement in their sensory properties and dietary value

pH effect on enzyme activity in wheat

In the present study optimum pH of the purified enzyme was examined. By using L-DOPA as substrate, the activity of purified PPO from wheat grains was measured at different pH values. The result is shown in Figure 1. It was studied that ideal pH for the purified enzyme was pH 5 in 10 cultivars. It was reported, that the optimum pH on which PPO shows maximum activity was 5 (Figure 1). The enzyme activity range was studied and it was noted that PPO was active between the pH range 4.5–9.0, and showed highest activity range at pH 5.0 while at pH 6 it showed half maximal activity. Its activity decreased speedily at pH 8.0. On the other hand, at pH 7.5 and 9.0, 50% of its activity was lost after 7.5 pH and no activity were noted when pH crossed the limit of 9 pH (Figure 1). Our results are similar to the results of Gao *et al.* [11]. The pH effect on PPO activity has been extensively studied in different plants, in most cases optimum pH was reported between 4.0 and 8.5, it was found that the pureness of enzyme and the form of buffer both have effect on the optimum pH [12, 13].

Figure 1. Showing activity of wheat PPO as a function of pH

Temperature influence on PPO and its stability towards temperature

To study the influence of different temperatures on PPO activity in wheat grain it was studied that action of PPO increases slowly from 10 to 20°C, above 30°C higher rate of activity was observed. The ideal temperature for PPO activity was also investigated and it was found that at 30°C PPO activity reaches to its maximum level (Figure 2). It was reported that when the temperature limit increased from 40°C, the activity started to decrease, and PPO

completely became inactive when temperature limits reached to 60°C and 70°C as shown in Figure 3. It has been reported by many authors that PPO is unaffected by temperature up to 90°C [14, 15]. Previously it has been reported that the temperature has effects between 10 °C and 80°C on PPO action of durum wheat. They further concluded that filtered durum wheat PPO has an ideal activity at 40°C. They also reported protein denaturation due to slow reduction in enzyme activity after 40°C temperature [16].

Figure 2. Showing activity of wheat PPO as a function of temperature

Figure 3. Activity of wheat PPO as a function of temperature stability. Each data point is the mean of two determinations. The vertical bars represent standard deviations

PPO activity assay with different substrates

Primary substrates of PPO are Phenolic compounds. In different plant sources PPO

shows different types and relative concentrations of natural phenols. To define the substrate specificity for the purified enzyme at pH 6, different substrates were studied and compared. The results are shown in Table 1. The PPO shows highest activity for L-DOPA because it was oxidized to L-tyrosine and diphenols. Chemical nature of the given substrate has great effect on PPO activity (Table 1). Different substrates show different oxidising rates for PPO and therefore divided into three categories i.e., (a) unoxidizable, (b) slowly oxidizable (c) readily oxidizable. Hydroquinone, Resorcinol, Phloroglucinol, *p*-coumaric acid, Ferulic acid and Vanillic acid were not good substrates and were unoxidizable substrates, while in the present study L-Tyrosine, Pyrocatechol showed slowly oxidizable substrate level activity. On the other hand 4-Methylcatechol, Caffeic acid, Chlorogenic acid, (+) Catechin, (-) Epicatechin, phenol were found to have readily oxidizable substrate level activity, while the most potent oxidizable substrate was L-DOPA. In a research work it was studied that, PPO extract from wheat bran shows differences in the oxidation rates for different phenolic substrates, it was also reported that some of them like ferulic acid, caffeic acid and chlorogenic acid are slowly oxidizable phenolics while 3-phenyl-2-propenoic acid was characterized as unoxidizable [17].

According to one study, different substrates like, L-DOPA phenol, catechol, caffeic acid methylcatechol and tyrosine when tested for best substrate, catechol and L-DOPA were found to be the best substrates [18]. Many names are given to Polyphenol oxidase because various substrates are catalysed by it, the top central of which are epicatechin, catechin L-DOPA, L-tyrosine, chlorogenic acid, methylcatechol, and pyrocatechol, among all of these the best substrates are L-DOPA and catechol [19, 18].

pH stability

The (Figure 4) shows dependence of PPO activity on pH. It has been standardized at pH 4.0 to 8.5 over a time of 4 days by utilizing substrate L-DOPA. It was shown in (Figure 4) that PPO enzymatic activity of wheat at pH 5.5 was the most interesting; it recommends that the compound is exceptionally unsteady at increase of pH, but no action subsequently at pH of 8.5 for 1 day, and a significant decrease in action of enzyme was noted at pH 8.0 (Figure 4). The outcomes of the present study are like Ziyan and Turk [20] who reported the ideal pH series was observed to be 4.5-8.2 for PPO of plants. In another study it was explained that the ideal pH for PPO of durum wheat was observed to be 6.5. Their outcomes reported that the best pH of durum wheat PPO is like PPOs from different sources. PPO of durum wheat was observed to be stable in the pH from 6.0 to 6.8 [16].

Figure 4. Activity of wheat PPO as a function of pH stability. Each data point is the mean of three determinations. The vertical bars represent standard deviations

Effect of SDS on PPO extraction and activity

The current research has additionally shown that PPO of wheat activity is affected by solid SDS which is anionic detergents. Figure 5 shows the SDS effect on PPO extraction and activity. SDS extraordinarily improved extraction of wheat PPO up to 50 mM while activity diminishes steadily as dose increases (Figure 5). PPO activity is present mostly in the external coatings of wheat portions (grain), and little activity is available in

processed flour [21-22]. In this manner, it is hard to measure these compounds with particular range of spectrophotometry utilizing typical substrates, for example, L-DOPA, catechol, phenol, tyrosine, and methyl catechol [18]. Subsequently an impact of the solid anionic detergents SDS on PPO action from a few other species has been depicted before and a current report likewise demonstrates that this detergents has impact on PPO activity removed from wheat and its action increases with SDS [23].

Figure 5. Showing effect of SDS on PPO activity in different wheat cultivar

Effect of inhibitors on activity of PPO

The impact of inhibitors on PPO activity has been contemplated since the primary reviews on PPO. Few inhibitors have been studied yet, in the present study, a number of inhibitors have been tested and it was found that the most weak inhibitors were Trans-cinnamic acid, Diethyldithiocarbonate, p-coumeric acid, Ferulic acid, Vanillic acid and DDT (Table 2). While the EDTA and ascorbic acid were the strongest inhibitors and Cysteine was the least potent inhibitor. Sodium Chloride, Sodium bromide Isoproterenol and sodium iodide *O*-phenanthroline were proved to be the effective inhibitors (Table 2). Our results are similar to the study of Madani *et al.* [24] and

Gao *et al.* [11], who demonstrated that the PPO activity from *Cleome gynandra* was marginally expanded by 0.1 percent SDS, intensely hindered by Hg^{2+} and Pb^{2+} , and totally repressed by 1.0 mM of ascorbic acid, L-cysteine, b-mercaptoethanol, sodium diethyldithiocarbamate and thiourea, and by 10 mM of dithioerythritol, sodium sulphite and sodium metabisulphite. According to the findings of Queiroz *et al.* [13] the PPO obtained from cashew apple (*Anacardium occidentale* L.) was reported that sodium chloride increase PPO action while ascorbic acid, citrus extract, sodium sulphite and sodium metabisulphite diminished PPO activity.

Table, 2. Effect of inhibitor on purified wheat PPO

Inhibitor	Relative activity inhibition (%)
Ascorbic acid	100
Diethyldithiocarbonate	0
2,2-dipyridyl	88.7
<i>O</i> -phenanthroline	72.2
Thiourea	15.9
Sodium azide	11.2
EDTA	99.1
Cysteine	50.1
Glutathione, reduced form	63.6
Iodoacetic acid	25.4
Sodium fluoride	9.3
Sodium Chloride	73.8
Sodium bromide	78.8
Sodium iodide	75.0
<i>Trans</i> -cinnamic acid	0
<i>p</i> -coumeric acid	0
Ferulic acid	0
Vanillic acid	0
ethyl alcohol	11.2
DDT	0
Isoproterenol	55.7

Effect of organic compounds on PPO extraction and activity

To focus on the effect of ketones on activity of PPO, study showed that Acetone from 1 to 20 percent in the extraction buffers had little effect on PPO activity in grain from ten wheat cultivars (Figure 6) and with just a minor increase at 40 percent in. To test the Alcohols effects on MOPS Buffer on activity of PPO, four alcohols (Ethanol, Methanol, 2-propanol and n-butanol) were examined independently. The aftereffects of PPO action in the liquor extraction are presented in Figure 6, 7, 8, 9, 10. Methanol had ignorable impact on PPO action. Indeed,

there was a minor diminishing in the activity as the amount of methanol was increased, but at 40 percent rise in PPO activity was noted (Figure 6). There was no observable effect on PPO activity when ethanol up to 20 percent was used (Figure 7). Ethanol and 2-propanol extracts carried on in fact a similar way, with unremarkable rise in the PPO activity at 40 percent fixation (Figure 8, 9). The maximum noteworthy effect of alcoholic amount on PPO activity in wheat grain was noted by using n-butanol. The particular increase in the activity of PPO was seen in n-butanol for all wheat cultivars (Figure 10). Our outcomes are similar with Okot Kotber *et al.* [17].

Figure 6. Showing effect of organic solvent (acetone) on PPO activity in different wheat cultivar

Figure 7. Representing effect of organic solvent (methanol) on PPO activity in different wheat cultivar

Figure 8. Represents effect of organic solvent (ethanol) on PPO activity in different wheat cultivar

Figure 9. Effect of organic solvent (isopropanol) on PPO activity in different wheat cultivar

Figure 10. Effect of organic solvent (n-butanol) on PPO activity in different wheat cultivar

Conclusion

The existing research study was completed to study the interesting biochemical characteristics of PPOs in wheat grains. Biochemical characterization of wheat PPO extracted from different wheat cultivars of Pakistan. It was examined that best substrates for PPO was L-DOPA, while EDTA and ascorbic acid were the strongest inhibitors. Further it was found out that optimum pH was 5 and at 30°C of temperature, PPO activity reaches to its maximum. In the light of our research findings, it seems possible that these proteins are mostly layer bounded. The extraction of PPO can be impressively

enhanced by utilizing generally larger amounts of SDS. Furthermore, PPO separated from high-activity wheat lines without detergent can be enhanced by SDS. The extraction of PPOs activity could be increased by adding 2-propanol, methanol, acetone, ethanol, and n-butanol in the extraction buffer.

Authors' contributions

Conceived and designed the experiments by N Taj & SZ Shah, Performed the Experiments by N Taj, Analyzed the Data N Taj, Contributed reagents/ materials/ analysis tools: N Taj & SZ Shah, Wrote the paper: N Taj.

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