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Purification and Characterization of Peroxidase from Broccoli (*Brassica oleracea l. Var. Italica*) Stems

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Research Article

Abstract

Background: Enzyme activity was increased during purification which could exert beneficial effect on plants.

Methods and Findings: Enzyme purification included extraction, (NH4)2SO4 precipitation, dialysis followed by sequential chromatographies with Sephadex G-75 and Sephadex DEAE A-25. The purified enzyme was characterized with time, pH stability, metal ions, thermostability, and substrate kinetics was determined using guaiacol as substrate.

The purification fold for purified broccoli stem peroxidase was 72.83 with 1.5% yield. The optimum time for enzyme activity was 6 min. and it remained stable between pH 4 to 8 having optimum pH of 6 using guaiacol as substrate. The enzyme showed maximum activity at 30°C and remained stable upto 50°C. The Km value of Broccoli Peroxidase was 0.35 m.mol/ml and 33 U/ml for guaiacol substrate by using Lineweaver-Burk graph and similarly using Michaelis Menten graph values was 0.34 m.mol/ml. Metals such as Na^{+}, Ca^{2+}, K^{+}, Mg^{2+} and Zn^{2+} exhibited no effect on enzyme activity.

Conclusion: These properties recommend that peroxidase could be auspicious tool for various applications in different analytical determination as well as in treatment of industrial waste.

Keywords: Peroxidase; *Brassica oleracea L. var. italic*; Chromatography; Dialysis; Purification.

1. Introduction

Peroxidases are large group of enzyme family called oxidoreductase that catalyze the reduction of peroxides, such as hydrogen peroxide (H_2O_2) and are responsible for the oxidation of various organic and inorganic compounds [1]. Peroxidase binds with other substrates such as ascorbate and ferricyanides and break them into harmless substances by donating electrons. Peroxidase catalyzes the oxidation of phenolic compounds and aromatic rings which occur naturally in plant tissues.

Peroxidases represent group of specific enzymes, such as iodine peroxidase, glutathione peroxidase and NADH peroxidase as well as a wide range of non-specific enzymes simply called as peroxidases. The molecular weight of peroxidases ranges from 30 to 150 kDa [2]. Peroxidases are associated with cell wall biosynthesis of plants. Their activity increases with plant age, with mechanical grazes and after vaccination with viruses, so they provide host resistance mechanism to plants [3].

In research areas such as biochemistry, medicine, genetics, enzymology and physiology, Peroxidases as a major enzyme group have occupied specific position in heat processing of vegetables [4]. The analysis of this enzyme have not only showed its negative effect on flavor, color and degradation of pigments of vegetables but also have positive energetic effect on vegetarian food [5].

The wide range of plant peroxidases have been purified and studied from different sources, such as Oil palm oil, Orange peel, Turnip root, Olives, Wheat, Sweet potato tuber, Green asparagus, Melon , Strawberry, Apple, Papaya fruit, Vanilla bean, Spinach, Red cabbage and cabbage leaves, Red beet, Spanish broom, Lettuce stem, Pearl millet, Sprouted green gram roots, Horseradish roots, Cauliflower, Drumstick tree leaves, Banana, Avocado, Rosemary leaves and Sweet gourd [6-32]. Multiple isoenzymes have been purified from all these sources which differ in their thermal stability, molecular mass, substrate specificity, pH optimization and their physiological role.

Peroxidases are also produced from microbial sources such as bacteria (*Pseudomonas* spp., *Bacillus subtilis, Bacillus sphaericus, Citrobacter* spp.), fungi (*Chrysosporium, Candida krusei, Coprinopsis cinerea, actinomycetes, phanerochaete*), Cyanobacteria (*Anabaena* spp.) and yeast (*Pichia*

pastoris) [33] have various industrial applications [2]. Despite the purification of peroxidase from these sources, one of the most important source rich in peroxidase is broccoli (*Brassica oleracea l. Var. Italica*). Broccoli is dicotyledonous plant vegetable belong to the family Brassicaceae or Cruciferae. Peroxidase purified from the broccoli (*Brassica oleracea. L. var. italica*) processing waste (mostly stem) have many clinical and industrial applications [34].

2. Materials and Methods

2.1 Study design

This research work was carried out at the Lab of Institute of Molecular Biology and Biotechnology (IMBB) and the Centre for Research in Molecular Medicine (CRiMM) at The University of Lahore.

2.2 Sampling

Fresh broccoli *(Brassica oleracea l. Var. Italica*) was purchased from a super market in Lahore.

Sample preparation: Broccoli stems and florets were separated by cutting and only stems were washed with distilled water. Juice of broccoli stems was extracted of about 100 ml by using juicer machine and homogenized with 200 ml of 0.1 M sodium-phosphate buffer, pH 7.0, in a ratio 1:2 respectively in a beaker. The homogenized extract was centrifuged at 5000 rpm for 20 min. and only supernatant was used for further purification. Supernatant was filtered using wet Whatman filter paper no.3 for further purification. A clear purified solution of broccoli extract was stored at -20°C for further study.

2.3 Enzyme assay

The activity of peroxidase enzyme was determined by the method described by Faizyme Laboratory Manual and Zia [35].

Lowry protein assay or Hartree-Lowry assay: Method: 50 0µl (0.5 ml) of crude sample was added from the pure extract of broccoli in 5000 µl (5 ml) of alkaline copper solution. The solution was placed at room temperature for 10 minute. Then 500 µl (0.5 ml) of Folin's reagent solution, diluted with distilled water in 1:1 ratio was rapidly added, mixed well and placed for 30 minutes at room temperature. Absorbance was noted at 600nm wavelength by using distilled water as blank on spectrophotometer [36].

3. Ammonium Sulfate Precipitation

Peroxidase (POD) enzyme was precipitated by ammonium sulfate $(NH_4)2SO_4$ salt for partial purification of enzyme. Different concentrations of 30%, 40%, 50%, 60%, 70% and finally 80% were prepared. The suspension was stirred on magnetic stirrer for two hours at room temperature. After adequate shaking, the solution was centrifuged at 6,000 rpm for 20 min and resulting supernatant and pellet were separated. Pellet was dissolved in 1 ml of 0.1 M phosphate buffer (pH 7) and enzyme assay of both the pellet and supernatant was performed using spectrophotometer following above mentioned method [37].

4. Dialysis

Maximum amount of enzyme obtained in 80% concentration of ammonium sulfate precipitation was dialyzed for desalting using dialysis bag also called as dialysis membrane. Before dialysis, the dialysis membrane was boiled in 0.1 M sodium carbonate (Na_2CO_3) till boiling point of solution and then retained the solution containing dialysis membrane for overnight. After that, precipitated sample was poured in dialysis bag and it was dialyzed against 200 ml, 0.1 M phosphate buffer (pH 7) on magnetic stirrer for 2 hours [38].

5. Gel Filtration Chromatography

Sephadex G 75 was used for gel filteration chromatography. After overnight soaking in water, gel was poured in the column (1 × 30 cm) and wait until it settled down. The packed column was washed with 0.1 M phosphate buffer (pH 7). After washing, 4 mL of dialyzed extract was applied on the column and fractions each of 4 ml were collected by pouring 0.1 M phosphate buffer (pH 7). Each fraction was then assayed for enzyme activity and the fraction containing maximum peroxidase activity was assayed for total protein estimation [39].

6. Ion Exchange Chromatography

After soaking, Sephadex DEAE A-25 was poured into the column (1 × 30 cm) and wait until gel get settled down. The buffer was removed and the packed column was washed first with 0.05 N HCl and then with 0.05 M NaOH solutions each of 100 ml. After washing, fractions from gel filtration chromatography containing maximum amount of peroxidase were pooled and applied in the column. Different molar concentration solution of NaCl ranging from 0.1 M to 1.0 M were applied and fractions were collected each of 5 ml. Each fraction was than assayed for enzyme activity and only the fraction containing maximum amount of peroxidase was assayed for total protein estimation [40].

7. Characterization

7.1 Effect of time

The influence of time on activity of broccoli peroxidase was determined by giving different

intervals of time such as after 3, 6, 9, 12 and 15 minute. Enzyme activity was determined after adding enzyme to the substrate for these different intervals of time and then absorbance was measured.

7.2 Effect of PH

Peroxidase activity was analyzed in the range of pH 4–9 using following buffer:

Na-acetate buffer (pH 4, 5), Na-phosphate buffer (pH 6, 7) and Tris-HCl buffer (pH 8, 9). The activity assay included H_2O_2, guaiacol and purified fractions of enzyme. The pH stability was investigated by measuring the activity of enzyme after buffer substrate solution had been kept overnight in various pH conditions and then the activities were tested under standard conditions.

7.3 Effect of temperature

The impact of temperature on broccoli peroxidase activity was determined by using 0.1 M phosphate buffer (pH 6). The reaction mixture of buffer substrate and enzyme extract was incubated at various temperatures such as 30, 40, 50, 60, 70 and 80°C for 6 minute in a thermostatic water bath. Enzyme activity was determined at these temperatures using spectrophotometer.

7.4 Determination of Km and Vmax:

Different concentrations of guaiacol ranging from 0.2 m.mol/ml to1.2 m.mol/ml were mixed with fixed saturated concentration of H_2O_2 along with 0.1M phosphate buffer and 40 µL of purified broccoli peroxidase. The solution was incubated and enzyme assay was performed. Vmax and Km values were determined for peroxidase using Lineweaver-Burk graph and Michaelis Menten graph. The kinetic data was analyzed by the LineweaverBurk plots and Michaelis Menten graph. Reaction was performed at room temperature.

7.5 Effect of metal Ions

The effect of various metal ions on the enzyme activity was verified by addition of 10 mM chloride salt of Na+ and K+ for monovalent and Mg^{2+}, Ca^{2+} and Zn^{2+} for divalent. The activity of peroxidase was determined after 6 min incubation under standard assay conditions in the presence of metal ions.

8. Results

8.1 Crude extract

The crude sample of broccoli along with 0.1 M phosphate buffer (pH 7) was exposed to spectrophotometric exploration at 470 nm wavelength in order to obtain enzyme activity, which is given in Tables 1-3.

Table 1. Enzyme activity of crude sample of Broccoli peroxidase at pH 7.

Enzyme fraction	Absorbance (OD)	Activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)
Crude enzyme	2.804	13.1788	1.999	6.592

Table 2. Enzyme activity of supernatant after ammonium sulfate precipitation.

Ammonium sulfate Concentration (%)	Absorbance (OD)	Activity (U/ml)
30	4.605	21.64
40	1.802	8.46
50	4.112	19.32
60	1.144	5.37
70	1.993	9.36
80	1.136	5.33

Table 3. Enzyme activity of pellet after ammonium sulfate precipitation.

Ammonium sulfate Concentration (%)	Absorbance (OD)	Activity (U/ml)
30	0.554	2.6038
40	2.078	9.76
50	2.745	12.878
60	8.319	39.09
70	8.828	41.49
80	12.78	60.06

Enzyme fraction	Absorbance (OD)	Activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)
Dialysis	11.32	53.204	1.20	44.3367

Table 4. Dialysis of 80% ammonium sulfate precipitated solution.

8.2 Ammonium sulfate precipitation

Different concentration of ammonium sulfate salt added in crude sample of broccoli to precipitate the proteins. Addition of 80% $(NH_4)_2SO_4$ salt could precipitate maximum amount of total protein in crude sample. Hence, appropriate broccoli peroxidase precipitation was found at 80% $(NH_4)_2SO_4$. Maximum activity was 60.06 U/ml in 80% $(NH_4)_2SO_4$ which directs the presence of enzyme (Tables 2 and 3).

8.3 Dialysis

80% ammonium sulfate precipitated solution was dissolved in 0.1M phosphate buffer (pH 7) dialyzed against the same buffer (Table 4).

8.4 Gel filtration chromatography

Dialyzed sample was subjected to sephadex G-75 for gel filtration chromatography. Out of 25 fractions 5^{th} one showed the maximum enzyme activity of 26.818 U/ml (Table 5).

Table 5. Determination of broccoli peroxidase activity through analysis of gel filtration chromatography.

Enzyme fraction	Absorbance (OD)	Activity (U/ml)
1	0.014	0.0658
2	0.006	0.0282
3	0.002	0.0094
4	1.899	8.925
5	5.706	26.818
6	4.62	21.714
7	4.596	21.601
8	2.832	13.31
9	2.64	12.408
10	2.11	9.917
11	1.73	8.131
12	0.593	2.787
13	0.583	2.7401
14	0.522	2.594
15	0.425	1.997
16	0.392	1.842
17	0.272	1.278
18	0.16	0.752
19	0.042	0.197
20	0.02	0.094

Table 6. Analysis of ion exchange chromatography for activity of broccoli peroxidase.

Enzyme fraction	Absorbance (OD)	Activity (U/ml)
1	0.019	0.0893
2	0.011	0.0517
3	0.725	3.4075
4	1.295	6.0865
5	1.884	8.8548
6	2.554	12.003
7	1.034	4.859
8	0.211	0.9917
9	0.026	0.122
10	0.012	0.564

8.5 Ion exchange chromatography

Fraction of gel filtration chromatography having maximum enzyme activity was subjected to sephadex DEAE A-25 for ion exchange chromatography. Out of 20 fraction 6th (0.6M NaCl) showed maximum activity of 12.003 U/ml (Tables 6 and 7).

8.6 Characterization of peroxidase

Effect of time: The effect of time on broccoli peroxidase is showed in Table 8. Maximum activity was obtained after 6 minute of incubation of about 14.053 which started decline after 6 minute, after that no change in activity was noticed (Table 8).

Effect of pH: The purified broccoli peroxidase, assayed at different pH ranging from 4 to 9, exhibited higher activity over a broad pH range (pH 4 to 7), with maximum activity around pH 6. Broccoli peroxidase was stable in the pH range of 4.5–7.5 (Table 9).

Effect of temperature: Broccoli peroxidase was assayed at optimum pH in different temperatures, ranging between 30 to 80°C for 6 minute. The enzyme showed maximum activity at 30°C and remained active at 50°C (Table 10).

8.7 Effect of substrate

Effect of metals ions: The effect of metal ions on broccoli peroxidase activity is presented in Table 11 and Figure 1. The enzyme activity was enhanced by K^+, $Ca2^+$, Na^+, Mg^{2+} and Zn^{2+} and no change in broccoli peroxidase activity was noticed with divalent cations such as Mg^{2+}, Zn^{2+} and monovalent ions Na^+, K^+ reported that peroxidase activated upto 50 mM (Table 12), while higher concentration inhibited the enzyme in a concentration dependent manner. During incubation for 6 minute Mg^{2+} and Zn^{2+} change the color of buffer substrate solution along with

Table 7. Summary of broccoli (*Brassica oleracea l. Var. Italica*) peroxidase purification.

Sample	Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Purification fold	Percentage yield (%)
Crude sample	13.17	1.998	6.592	1	100
Ammonium sulfate Precipitation	60.06	2.615	22.96	3.48	22.80
Dialysis	53.204	1.2	44.33	6.72	9.42
Gel filtration Chromatography	26.818	0.189	141.89	21.52	3.391
Ion exchange Chromatography	12.003	0.025	480.12	72.83	1.5

Table 8. Effect of time on broccoli peroxidase activity.

Serial.No.	Reaction Time (min)	Absorbance (OD)	Enzyme Activity (U/ml)
1	3	2.153	10.119
2	6	2.99	14.053
3	9	2.86	13.442
4	12	2.783	13.0801
5	15	2.563	12.0461

Table 9. Effect of pH stability on broccoli peroxidase activity.

Serial No.	Buffer	Change in pH	Absorbance (OD)	Enzyme Activity (U/ml)
1	Na-acetate buffer	4	1.965	9.235
2	Na-acetate buffer	5	2.395	11.256
3	Na-phosphate buffer	6	2.554	12.003*
4	Na-phosphate buffer	7	2.404	11.298
5	Tris-HCl buffer	8	1.526	7.1722
6	Tris-HCl buffer	9	0.345	1.6215

Table 10. Effect of temperature on broccoli peroxidase activity.

Serial No.	Temperature (°C)	Absorbance (OD)	Enzyme Activity (U/ml)
1	30	4.498	21.1406
2	40	1.883	8.8501
3	50	0.955	4.4885
4	60	0.134	0.629
5	70	0.124	0.582
6	80	0.098	0.4606

Table 11. Effect of substrate (Guaiacol) on the activity of broccoli peroxidase.

Serial No.	Change in Substrate (Guaiacol) (mM)	Absorbance (OD)	Enzyme Activity (U/ml)
1	0.2	1.466	6.890
2	0.4	2.481	11.67
3	0.6	5.096	23.95
4	0.8	5.678	26.68
5	1	5.89	27.683
6	1.2	6.144	28.87

Table 12. Effect of metal ions on broccoli enzyme activity.

Serial No.	Metal Ions	Absorbance (OD)	Enzyme Activity (U/ml)
1	potassium chloride	4.039	18.93
2	sodium chloride	5.305	24.93
3	calcium chloride	5.422	25.48
4	zinc chloride	4.869	22.884
5	magnesium chloride	4.443	20.88

enzyme solution from brown to milky but activity remain same.

9. Discussion

Results of this study revealed that broccoli is a good source of peroxidase with various isozyme forms. Peroxidase purified from the stem of broccoli has various industrial as well as pharmaceutical applications.

Peroxidase activity in the crude extract of broccoli stem using H_2O_2, guaiacol and 0.1 M phosphate buffer (pH 7) was 13.178 U/ml which have similarity with the work of (41) who followed the same method of extraction and at neutral pH have enzyme activity of about 23.6 U/ml. as the activity of peroxidase increases it provide defense mechanism to plants.

Peroxidase activity was increased through ammonium sulfate precipitation and at 80% precipitation, peroxidase showed maximum activity of 60.06 U/ml and after dialysis the activity was reduced to 53.20 U/ml. Cauliflower (*Brassica oleracea L. var. botrytis*) belongs to the broccoli (*Brassica oleracea l. Var. Italica*) family showed maximum activity of about 69.56 U/ml during ammonium sulfate precipitation and 59.44 U/ml after dialysis which is closed to broccoli PODs activity following unit definition which

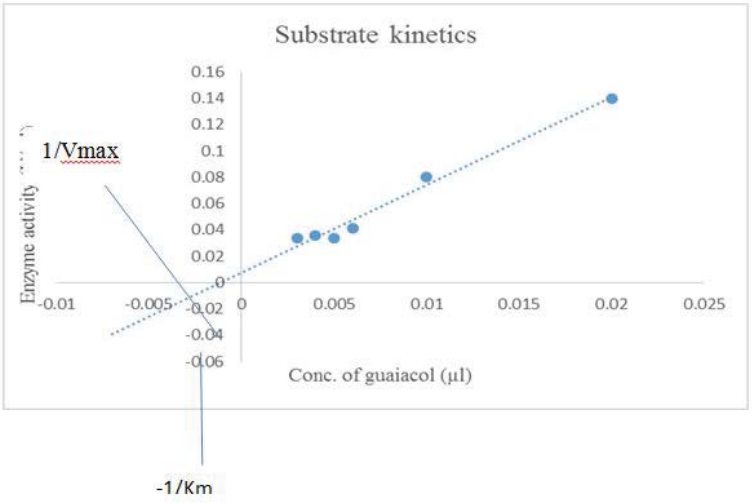

Figure 1. Lineweaver-Burk plot for Broccoli peroxidase under varying substrate (Guaiacol) concentrations indicating the *K*m and *V*max values.

describe one unit of enzyme activity per micromole or microgram of substrate. Ammonium sulfate precipitation leads to partial purification of enzyme which is further purified through dialysis [27]

The purification fold for purified broccoli stem peroxidase after gel filtration and ion exchange chromatography was 72.83 with 1.5% yield. The protein fold was higher with that [41, 42] work who gained 30.6 fold protein with 2.6% yield after purification, while it was lower than red cabbage (*Brassica oleracea var. capitata f. rubra*), turnip roots (*Brassica L.*) belongs to broccoli family and kmatsuna (*Brassica rapa*) who gained 120.6 fold protein with 2.9% yield, 69.13 fold protein with 9% yield and 100 fold protein with 8% yield respectively [19, 21, 43].

Broccoli peroxidase was characterized with various parameters such as time, pH stability, thermostability, substrate specificity and effect of various metal ions. Maximum enzyme activity was obtained after 6 min of incubation which remained stable with pH range of 4 to 7 and had optimum pH 6, the enzyme purified from the turnip roots [21, 34], cauliflower [28], red cabbage [19] showed pH range from 4 to 6 with optimum pH of 5.5 to 6.

Broccoli peroxidase showed maximum activity at 30°C and activity was remained stable upto 50°C than activity started decline and at 80°C the activity was approximately nil. The work has similarity with the work of 21, 28, 19. Their purified enzyme was stable at 30°C and activity remained stable upto 60°C which was too low at 80°C.

The Km and Vmax values of bPOD were 0.35 m.mol/ml and 33 U/ml for guaiacol substrate respectively, by using Lineweaver-Burk graph and similarly using Michaelis Menten graph values were 0.34 m.mol/ml and 30 U/ml for both Km and Vmax respectively. From the previous study Km and Vmax value of red cabbage (Brassica oleracea var. capitata f. rubra) were 0.042 mM and 1.46 U/ml, respectively [19].

Effect of various metal ions was observed on peroxidase such as chloride of Na+, K+, Ca^{2+}, Mg^{2+} and Zn^{2+}. Enzyme activity was not affected with these monovalent and divalent metal ions which showed high stability of peroxidase. The work correlated with the work of Abdurrahman and Mohammad v [44] who purified peroxidase from date palm leave and have same results for metal ion stability on peroxidase, however, they showed Cd+ inhibitory effect on peroxidase.

10. Conclusion

Plants have been used for purification of enzymes to meet present day industrial demand. In present study a peroxidase enzyme was purified from broccoli stems. The purified enzyme was characterized with time, pH stability, metal ions, thermostability, and substrate kinetics was also determined using guaiacol as substrate. The properties of this enzyme recommend that peroxidase could be auspicious tool for various applications in different analytical determination as well as in treatment of industrial waste.

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