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PURIFICATION AND SOME PARTIAL CHARACTERIZATION OF PEROXIDASE ISOENZYME FROM *BRASSICA OLERACEA CAPITATA* L.

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Abstract

Acetone fractionated peroxidase from crude extract of *Brassica oleracea* leaves (Cabbage) was purified in three steps on chromatographic columns, using Sp-Sepharose, DEAE-Sepharose and Con A-Sepharose. The specific activity of purified main isoenzyme (BOC-POD) is 1887 u/mg protein with RZ: 3.1, which is 172 times more than the RZ of crude extract with 4.3% recovery. The molecular weight of BOC-POD is about 45,000 Dalton. Maximum pH, thermal activity and stability of this purified enzyme are also determined. Km of this isoenzyme was measured by Linewearver-Burk curve for O-dianisidine towards H_2O_2. This purified enzyme could be used in manufacturing diagnostic kits.

Keywords: *Brassica*; Cabbage; Purification; Peroxidase

Introduction

Peroxidase (donor: H_2O_2 oxidoreductase, EC: 1.11.1.7) is distributed in a wide range of plant species, in multiple molecular forms. Many researchers have worked on different aspects of peroxidase. Peroxidase has been implicated in metabolic processes such as ethylene biogenesis, cell development and membrane integrity [1]. Its properties and physiological roles in fruits and vegetables have been reviewed by several authors. Many studies have been done on amino-acid sequencing and heme structure of peroxidases [2,3].

Several isoperoxidases, notably horseradish and turnip, have been studied in great detail during the past two decades [4]. Studies in our laboratories and by other workers on peroxidase isoenzymes from many different plants indicated that physical and kinetic properties and substrate preference of this isoperoxidase even from a single source might vary significantly [5-8]. In addition, it is generally accepted that peroxidase activity and its isoenzyme patterns alter with changes in plant development.

From the economical point of view, peroxidase is an important enzyme. It is used in diagnostic kits for enzymatic determination of glucose, uric acid, cholesterol and many other metabolites in biological fluids and is also an important enzyme in ELISA systems.

To find a locally available and economical source of

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peroxidase, we have already studied purification and characterization of peroxides from *Raphanus sativus* L. var. *cicil* [7] and *Raphanus martimus* Sm. [8]. In the present study, the affinity chromatography method with high efficiency for purification of peroxidase from *Brassica oleracea capitata* L. (cabbage) and for the first time some properties of purified peroxidase from cabbage are presented.

Experimental

All the enzymes and chemicals were purchased from Boehringer Mannheim.

Extraction of Crude Enzyme

Cabbage leaves (*Brassica oleracea capitata* L.) was purchased at local markets, in winter. It was identified as *Brassica oleracea capitata* L. by Dr. Gh. Amin in Herbarium of Pharmacognosy Department of faculty of pharmacy, Tehran University of Medical Science. All purification procedures were carried out at 20-25°C. The leaves were washed, cut into small pieces and frozen at −20°C for 24 h.

After thawing, leaves were homogenized with 1/2 W/V K_2HPO_4 solution (0.1 M). The homogenate was filtered through chess0 clothe and then centrifuged for 10 min at 10,000 rpm with Centrifuge: Tomy Seiko Co., Ltd, Model: RS_20 IV, to remove debris. The clear centrifuged extract was designated as crude soluble enzyme.

Peroxidase and Protein Assay

O-dianisidine was used as a substrate to measure peroxidase activity spectrophotometrically at 460 nm using Shannon method [9]. One unit of enzyme will form O° from 1 µM H_2O_2 in 60 Sec at pH: 5.4 at 30°C.

Protein concentration was measured according to Bradford method [10]. During purification steps by ion-exchange and Con A-Sepharose columns, absorbance at 280 nm was used as an indicator for protein concentration.

Acetone Fractionation

The crude enzyme was treated with 1 volume cold (−20°C) acetone and left for 6 h at refrigerator (−20°C), then centrifuged for 10 min at 10,000 rpm. The supernatant was removed and another volume of cold (−20°C) acetone was added to it and kept at +4°C over night. The precipitated proteins were collected by centrifugation at 10,000 rpm for 10 min and dissolved in acetate buffer 10 mM, pH:4.5.

Sp-Sepharose Chromatography

It was carried out on Pharmacia Sp-Sepharose in a column (8×150 mm) with acetate buffer, 10 mM, pH: 4.5. Proteins were then eluted with a stepwise gradient of 10ml of this buffer consisting of 0, 2, 4, 6, 8, 10, 12 mM NaCl at flow rate of 18 ml h^{-1}. Working volume of each fraction was 3 ml.

DEAE-Sepharose Chromatography

It was done on Pharmacia DEAE-Sepharose in a column (8×150 mm) with 10 mM tris buffer pH: 8.5. Absorbed proteins were eluted with stepwise gradient of 10 ml of the same buffer consisting 1, 2, 3…15 mM NaCl at flow rate of 18 ml h^{-1}. Three ml_Fractions were collected.

Con A-Sepharose Affinity Chromatography

It was done on Pharmacia Con A-Sepharose in a column (8×50 mm) with phosphate buffer, 50 mM, pH: 6, containing 1 mM of $CaCl_2$, NaCl, $MgCl_2$, $MnCl_2$. The proteins were eluted with the same buffer containing 0.2 M sucrose.

SDS-PAGE Analysis

All of the fractions were tested for molecular weight estimation and homogeneity by 12% SDS poly-acrylamide as described by Hames [11].

Protein samples was mixed with 0.5 volume sample buffer and boiled for 2-5 min, then were applied in the gel at a constant current about 3.5 mA per 1 cm of the gel.

Gel was stained by silver nitrate [11]. The molecular weight of proteins was estimated using Pharmacia Low Molecular Weight size marker.

Activity of BOC-POD in Different pHs and Temperatures

Activity of purified peroxidase (BOC-POD) was measured in pHs (3.5-9) using 0.05 M buffers of sodium acetate for pH ranging from 3.5-6.5 and Tris-base for pH ranging from 7-9.

BOC-POD (150 u/ml) incubated at 37°C in this buffer as the method of enzyme assay [9] and the activity was measured and compared with the initial activity.

Activity of BOC-POD (150 u/ml) in different

temperatures (30-70°C) was estimated as the enzyme assay [9].

Stability of BOC-POD in Different pHs and Temperatures

Determination of pH stability of BOC-POD (150 u/ml) was performed with the same buffer as pH-activity. POD was incubated for 4 h at 37°C, and then enzyme activity was measured as Shannon method [9].

For measuring of thermal stability of BOC-POD, enzyme (900 u/ml) in acetate buffer (50 mM, pH: 5.4) was incubated for 30 min in different temperatures, then proportion of remained activity was compared with the initial activity. Enzyme assay was performed as Shannon method [9].

Km Determination

Michaelis-Menton constant for the peroxidase towards substrate O-dianisidine and H_2O_2 was determined by incubating BOC-POD (150 u/ml) during one minute with varying concentrations of H_2O_2 and fixed saturation concentration of O-dianisidine, then for the best conclusion, we used Lineweaver-Burk plots to determine Vmax and Km.

Enzymatic Determination of Cholesterol and Uric Acid

Cholesterol was determined enzymatically by an assay reaction mixture containing 0.05 M phosphate buffer, pH: 7, 30 mM phenol, 0.8 mM aminoantipyrine, 0.2 u/ml cholesterol oxidase, 0.2 u/ml cholesterol esterase, and 1.5 u/ml purified peroxidase enzyme (POD-BOC). The reaction was started by the addition of 10 µl of cholesterol standard B.M. The changes in absorbance were measured at 500 nm after 10 min incubation at 37°C against a blank containing 10 µl of water instead of cholesterol.

In uric acid determination the same buffer was used with uricase 0.3 u/ml instead of cholesterol oxidase and cholesterol esterase. The reaction volume and assay condition was the same.

Results

Enzyme Purification

Extracted solution of 300 g cabbage leaves was fractionated by cold acetone (−20°C). Precipitation of two volume acetone was dissolved in 28 ml acetate buffer (pH: 4.5, 10 mM). This solution (310 u/ml) was first dialyzed against acetate buffer (pH: 4.5, 10 mM) and fractionated on a SP-Sepharose column (Fig. 1).

The chromatogram showed five sharp protein peaks. Peaks No.1 (fractions No. 1-11) and 3 (fractions No. 20-23) had peroxidase activity. Peak No.1 (Sp-1) was not absorbed to the column under these chromatographic conditions but had the highest peroxidase activity. In the SDS-PAGE analysis, peaks 1 and 3 were found to have protein impurities. Peak Sp-1 had about 65% of the total initial activity and therefore was selected for further purification. The second step of chromatography for this peak was performed on DEAE-Sepharose column after dialyzing the peak (33 ml, 170 u/ml) with 10 mM Tris buffer pH: 8.5. The elution profile is shown in Figure 2. One sharp protein peak was observed, DEAE-1, (fractions No. 9, 10, 11) in chromatogram with peroxidase activity but there were many protein bands in SDS-PAGE analysis. For further purification, DEAE-1 (9 ml, 208 u/ml) was loaded on Con A-Sepharose. The elution profile is shown in Figure 3. The elution profile showed two protein peaks, which were named peak A (fractions No. 5-8) and B (fractions No. 18-20) respectively. Only peak B had peroxidase activity (5 ml, 151 u/ml) and was shown as a single protein band in SDS-PAGE electrophoresis with molecular weight about 45,000 Dalton (Fig. 4), so this eluent fraction was named BOC-POD. Results of all purification process have been shown in Table 1.

The specific activity of this purified enzyme was about 13 times more than the crude enzyme with RZ 3.1, which was 172 times more than the RZ of crude enzyme (Table 1).

Rz (Reinheistszahl) is absorbance ratio A_{403}/A_{275}. It is a measure of heme content.[9].

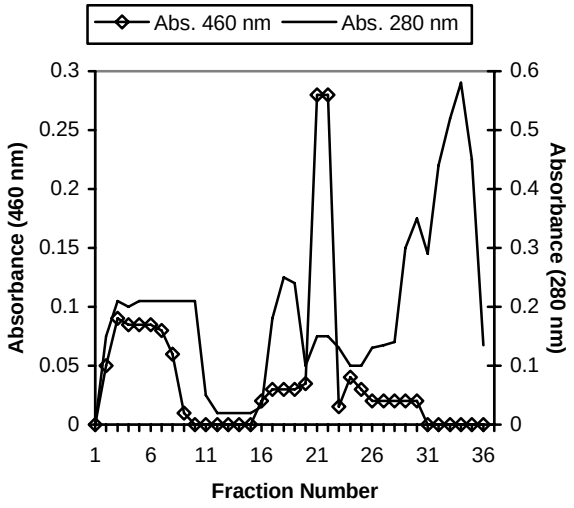

Figure 1. Elution profile for cabbage peroxidase on Sp-Sepharose.

Physico-Chemical Properties

Data concerning effect of various pHs on BOC-POD activity was examined at 37°C between pH: 3.5-9 using 0.05 M buffers. The initial activity of BOC-POD was about 150 u/ml (Fig. 5). The optimum pH for BOC-POD in this condition was about 4 in acetate buffer.

BOC-POD peroxidase activity in different temperatures was measured by incubating 150 u/ml of enzyme in temperatures ranging from 30-70°C (Fig. 6). The best temperature for highest activity of BOC-POD was 50°C.

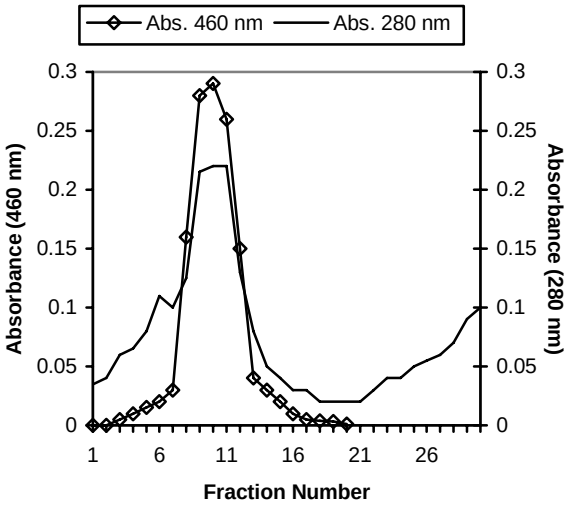

Figure 2. Elution profile for cabbage peroxidase on DEAE-Sepharose.

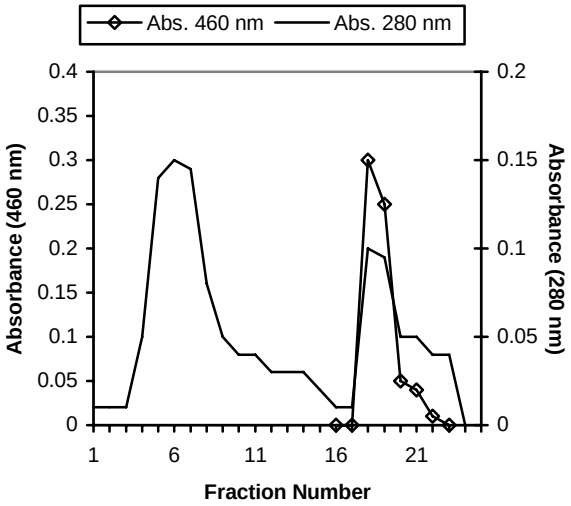

Figure 3. Elution profile for cabbage peroxidase on Con A-Sepharose.

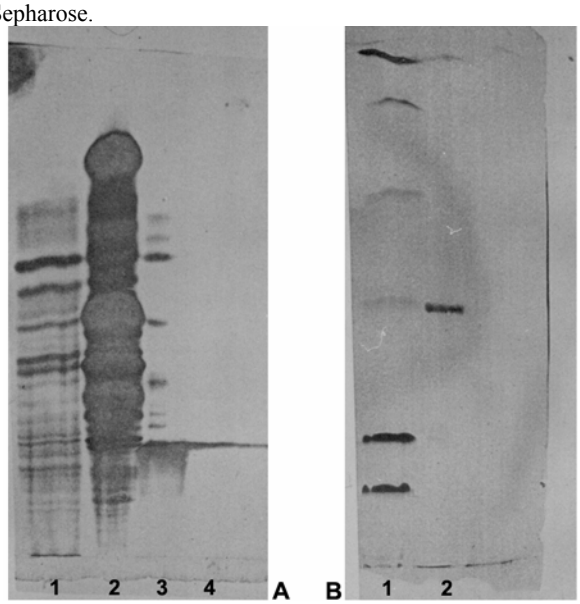

Figure 4. SDS-PAGE of various fractions of cabbage peroxidase during purification procedures. A: 1. Crude extract, 2. Acetone fraction, 3. DEAE-1, 4. BOC-POD; B: 1. Size Marker, 2. BOC-POD.

Figure 5 also shows pH stability of BOC-POD in 0.05 M buffers with pHs between 3.5-9. After four hours of incubation of BOC-POD (150 u/ml) in mentioned pHs at 37°C, the activity was assayed. The maximum stability for BOC-POD after four hours is in pH: 6. In pHs ranging 4.5-6, BOC-POD loses only 20% of its total activity. In higher pHs than 6 it loses more than 60-80% of its total activity.

Thermal stability of BOC-POD (900 u/ml) at

temperatures ranging from 30-60°C after 30 min was also measured. As it is shown in Figure 6, BOC-POD keeps more than 90% of its activity up to 50°C, but in higher temperatures it loses most of its activity.

At optimum pH (pH: 4), BOC-POD exhibits the typical linear Linewearver-Burk plot. Km and Vmax value 5 for BOC-POD are about 0.56×10^{-3} M and 7.3×10^{-3} M/min respectively.

To find the efficacy of purified BOC-POD for diagnostic application, cholesterol and uric acid of 20 serum samples were measured with prepared kits and results compared with the results of measurement with Boehringer Mannheim kits.

The results showed the efficiency of both kits with respect to sensivity, correlation with standard kits and precision for measurement of standard serum Precipathe® and Precinorm®. The correlation between the results was 99.1 and 99.8% for cholesterol and uric acid respectively.

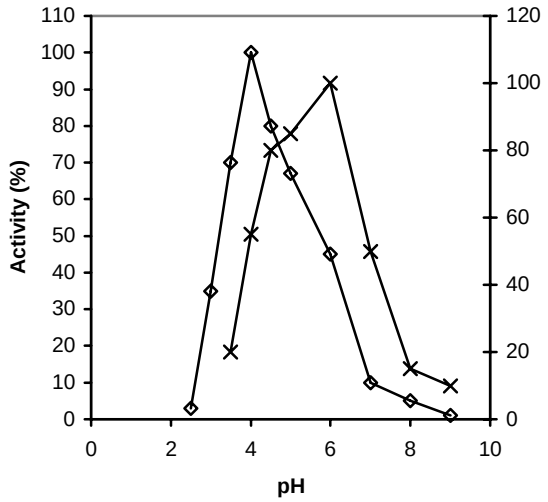

Figure 5. Effect of different pHs on the activity and on the stability of BOC-POD after four hours.

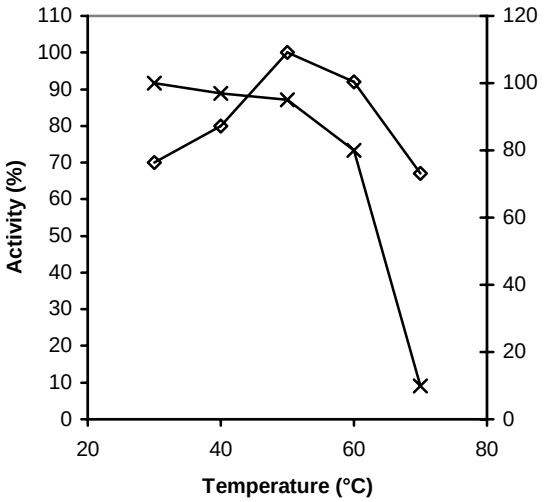

Figure 6. Effect of different temperatures on the activity and on the thermal stability of BOC-POD after 30 min.

Table 1. Fractionation protocol of *Brassica oleracea capitata* L. (cabbage) peroxidase

	Volume (ml)	Activity (u/ml)	Protein (µg/ml)	Specific activity (µ/µg)	RZ*	Total activity (u)	Total protein (mg)	Recovery** (%)	Purification Fold
Crude Extract	150	117	1.6	146	0.02	17550	240	100	0
Acetone Fraction	28	310	1.2	258	0.05	8680	33.6	49.5	1.7
Sp-1	33	170	0.29	586	0.08	5610	9.57	32	4
DEAE-1	9	208	0.17	1223	1.25	1872	1.53	10.7	8.4
BOC-POD	5	151	0.08	1887	3.1	755	0.4	4.3	13

* RZ: abs..403 nm / abs..275 nm

** Comparing to the crude extract

The results were also in agreement with the results of kits prepared with horseradish peroxidase with RZ: 3 from Bohringer Mannheim, and in fact there was no significant different between results. Moreover the blank solution – containing all reagents except cholesterol and uric acid – is colorless and quite clear. The rate of reaction, in presence of cholesterol and uric acid at 505 nm was equal to Bohringer Mannheim kits.

HRP with RZ: 3 from B.M. was used during past years for preparation of our enzymatic kits. The kits for determination of cholesterol and uric acid using either POD-BOC or B.M. HRP were found to be as efficient as each other.

Discussion

Several authors have studied on peroxidase. Schoenbein (1855) was the first who searched vigorously on peroxidase [12]. Fig peroxidase was the

first one isolated and investigated in 1936 by Summer and Howell [13]. Peroxidases have been purified from such diverse sources as horseradish, yeast, sweet potato, turnip, and wheat during 1942-1956 [14].

One of the first successful methods in purification and crystallization of purified peroxidase was performed by Shannon and his colleagues in 1966 [9]. He separated about 7 isoenzymes, from horseradish by ammonium sulfate precipitation and using ion-exchange chromatography. Delincee reported about 20 peroxidase isoenzymes by thin layer isoelectric focusing [15].

In 1968, Mazza and his colleagues worked on purification of turnip peroxidases and they separated about seven isoperoxidases [16].

Ion exchange and affinity chromatography have already been used for purification of peroxidase from horseradish [17]. In this work, after fractionation by acetone, main cabbage peroxidase (BOC-POD) was successfully purified by ion exchange and affinity chromatography. Comparing with crude extract, the recovery of purified enzyme was not very high, but purity, as was shown by SDS-PAGE analysis and RZ, was very high. Therefore this method may be useful for purification of peroxidase from sources with high protein content.

It has been reported that peroxidase isoenzymes from various sources have different molecular weights ranging from 30,000-60,000 Dalton [18], *e.g.* four isoenzymes of turnip peroxidase have molecular weights between 37,000 and 57,000 Dalton and 30,000-54,000 for Japanese radish isoperoxidases [16]. The molecular weight (MW) of BOC-POD was about 45,000 Dalton, which is in the range of MW of most the known peroxidases.

Peroxidases are mainly used in determination of metabolites with other enzymes, therefore it is required to have good thermal stability and activity in a wide range of pH. BOC-POD is more active and stable in acid pHs. Horseradish peroxidase is more active and stable in neutral pHs and therefore BOC-POD could be a suitable alternative whenever more stability and activity in acidic conditions are needed.

Optimum pH and temperature for cabbage peroxidase and its stability are comparable with other reports too [19-21]. For example, optimum pH and temperature for strawberry peroxidase are reported to be 6 and 30°C respectively. This enzyme keeps almost its original activity after heat treatment up to 45°C for 20 min at pH: 6.0-8.0 (22).

Comparing Km values of cabbage peroxidase with six isoenzymes of Korean radish shows that Cabbage has 0.5-1 times Km values comparing with Korean ones towards O-dianisidine (4).

Brassica oleracea capitata L. (cabbage) is a vegetable available almost in all seasons at very low price and large quantity in our country. Although peroxidase content of cabbage might not to be as high as horseradish peroxidase, most of its properties are similar to HRP and by this procedure it is possible to obtain highly purified peroxidase, suitable for diagnostic application.

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