Screening of peroxidase from various vegetables sources, partial purification, some biochemical properties and its antimicrobial activity

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فحص البيروكسيد يز من أنواع مختلفة من الخضروات و التنقية الجزئية و بعض الصفات الكيمو حيوية و فعاليتها ضد البكتريا

الخلاصة

تم استخلاص أنزيم البيروكسيد يز من أربع أنواع مختلفة من الخضروات هي الفجل ،الشلغم ،اللهانة و الفلفل الاخضر . الانزيم المستخلص تم تنقيته جزئيا بأضافة سلفات الأمونيوم و DEAE كروموتوغرافيا. وقد درس تأثير كمية كوايكول و بيروكسيد الهيدروجين و كل من الأس الهيدروجيني (pH) و درجة الحرارة على الأنزيم.حيث وجدت ان أعلى فعالية للأنزيم عند ما كانت ال pH ما بين (5 .6-7) بينما كانت أعظم فعالية للأنزيم في درجة حرارة 30 م. جربت فعالية الأنزيم المستخلص من الخضروات المنقاة ضد أنواع مختلفة من البكتريا و وجدت بأن فعالية الأنزيم ضد هذه الأنواع من البكتريا كانت ضعيفة.

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Abstract

The peroxidase enzyme was extracted from four different vegetables which were radish, turnip, cabbage and green pepper. The extracted enzymes were partially purified by ammonium sulphate and DEAE cellulose (Diethylaminoethyl cellulose) chromatography. The effect of amount of guaiacol, hydrogen peroxide, pH and temperature were investigated. The optimum pH for peroxidase activity was in the range (6.5-7.0), while optimum temperature for extracted enzymes was 30°c. The extracted enzyme from selected vegetables was tested for their antimicrobial activity against different species of bacteria. The enzymes show weak antibacterial activity towards the tested species of bacteria.

1.Introduction

Peroxidase (POD) is a heme protein, which is a member of oxidoreductases [E.C.1.11.1.7] catalyze the oxidation of a wide variety of organic and inorganic substrates using hydrogen peroxide as the electron acceptor. Peroxidases are widely distributed in living organisms including microorganisms, plants and animals [(1)].Plant peroxidases are known to be comprised of a range of isoenzymes differing in number from one vegetable source to another and have been observed to be differ with respect to thermal stability, optimum pH, substrate specifity, amino acid composition and their physiological roles in plant tissues [(2)].

POD is found in many plant-based foods. The enzyme is highly specific to its peroxide substrate, of which H_2O_2 is the most common, but it has low specifity toward its hydrogen donor substrate. In the presence of peroxide, PODs from plant tissue are able to oxidize a wide range of phenolic compounds, such as guaicol, pyrogallol, chlorogenic acid, catechin and catechol. Oxidation of a wide range of organic compounds has led to the speculation that the enzyme may be associated with losses in colour, flavor, and nutritional value of processed foods [(3)].

PODs from several plants have been purified and studied. These include, for example, tomato[(4,5,6)], peanut[(7)], Cox's apple[(8)], green pea[(9,10)], mango[(11)], wheat germ[(12)], tobacco[(13)], carrot root[(14)], melon[(15)], turnip[(16)], sweet potato[(17)], olives[(18)] and hot pepper[(19)]. In all cases multiple

isoenzymes have been reported. Isoenzymes from these various plant sources differ with respect to molecular mass, thermal stability, optimum pH and physiological role. From the economical point of view, POD is an important enzyme because it is used in diagnostic kits for enzymatic determination of glucose, uric acid, cholesterol and many other metabolites in biological fluids [20].The aim of the present study was partial purification of peroxidase in four vegetables sources then testing the antibacterial action of the extracted enzyme in different species of bacteria.

2. Material and Methods

2.1. Chemicals

All chemicals were of analytical reagent grade otherwise. Polvinylpyrrolidone was obtained from Fluka, UK. Guaiacol was purchased from Across, USA. Disodium hydrogen phosphate was obtained from Fisher Chemicals, UK. Potassium di-hydrogen phosphate, tri-sodium citrate, citric acid and phosphoric acid were purchased from Carlo Erba, Italy.

2.2. Instruments

The absorbance measurements were carried out by Cecil spectrophotometer model 3021. A Gerhard pH-meter was used for adjusting the acidity of the solutions.

2.3. Sample preparation

Radish, turnip, green pepper and cabbage samples were obtained from a local market in Erbil city. All plant samples were washed several times with tap water. About 200 g of each sample was homogenized by blender for 15 minutes. The samples were stored in refrigerator (4 °c) for further experiment [19].

2.4. Enzyme extraction

A 200 g of the chopped pieces of fresh vegetable were extracted with 400 ml of 0.1 M of phosphate buffer solution (pH=7.0) containing 2.0% polyvinyl pyrrolidone . The homogenate was centrifuged at 2000 rpm for 5 minutes .The superannuates was passed through Wattman no.42 filter paper. The superannuate was collected as enzyme extract [19]. Ammonium sulphate (solid regent) was added to extract to 40% saturation. The solution was centrifuged at 15000rpm for 20minutes. The supernatant was collected and its volume was measured, then ammonium sulphate was added to it again to 85% saturation. The mixture was centrifuged again at 15000rpm for 20 minutes the supernatant was collected and checked for enzyme activity .The precipitate was dissolved in 35 ml of distilled water and also checked for enzyme activity. The precipitated enzyme by ammonium sulphate step was dissolved in 35 ml distilled water then dialyzed over night against distilled water. The activity of enzyme was tested in the desalted extract [21].

2.5. Purification of POD by DEAE chromatography

The DEAE resin (powder) was rapidly swollen (in phosphate buffer) by heating the slurry at 90 °c for 5 hours using water bath. Appropriate volume of the slurry was poured in to the column in order to fill completely ,the require column bed height. After the column was packed to the desired bed height (i.e. 5 cm) layer of buffer was poured on its top with the help of pipette. For 5 cm bed volume, the column was washed with15ml of 0.5N of HCl solution. The column was then washed with distilled water until the effluent pH was 7 .0. The column was washed with 15 ml of 0.5 N of NaOH solution. The column was again washed with distilled water until the effluent was of pH 7.0 [21].

2.6. Screening for Antimicrobial Activity

The antimicrobial activities of the crude aqueous extracts of the various vegetables (Radish, turnip, cabbage and green paper) plant samples were evaluated by antibiotic well method. The

solidified LB agar plates were inoculated with 100 ml of gram –negative bacteria E.coli and two gram –positive bacteria, Bacillus subtilis and Staphylococcus aureus by swabbing. The wells were prepared on the agar plate already seeded with cultures (106 cfu/ml) with the help of a cork borer (10mm dia.). The wells were loaded with 200 µl volume of the extracts and 50 µl of tetracycline was used as a control. The plates were incubated at 37◦ C overnight. For each extract three replicate trials were conducted against each organism [22].

2.7. Peroxidase assay

The POD activity in selected vegetables was measured using guaiacol substrate .The procedure involves addition of 10 µl of enzyme extract to a mixture of 1 ml of 22.5 mM of hydrogen Peroxide and 1 ml of 45 mM of guaiacol . The final volume was adjusted to 3 ml by addition of 0.1 ml phosphate buffer solution (pH 6.0). The changes in the absorbance at 470 nm were measured for 3 minutes at 20 °c. One unite of enzyme activaity is defined as 0.01 change in absorbance at 470 nm [1].

2.8. Protein assay

The protein concentration in the extracted solutions was determined by procedure described by M.M. Braddford [23].

2.9. Effect of hydrogen Peroxide

The influence of hydrogen peroxide (22.5 mM) volume on POD activity was checked using the reaction system in which the volume of hydrogen peroxide solution changed and the final volume completed to 3.0 ml.

2.10. Effect of guaiacol

The effect of 45 mM of guaiacol volumes on POD activity was studied also; the reaction system was used in which the volume of guaiacol solution varied and the final volume were completed to 3 ml.

2.11. Effect of pH

The influence of pH on the enzyme activity was investigated in the range 3.0 -5.5 using 0.1 M citrate buffer solution and 0.1 M of phosphate buffer solution in the pH range 6.0 to 8.0 .The Effect of pH was carried out through a reaction mixture containing 10 µl of extracted in enzyme solution, 0.8 ml hydrogen peroxide solution(22.5mM), 1.2 ml of guaiacol solution(45mM) and 1 ml of 0.1 M various buffer solution [19].

2.12. Effect of temperatures

Peroxides activity was determined at various temperatures (10 -90 C) in a water bath. The substrate and buffer solutions were incubated for 5 minutes at various temperatures before adding of the enzyme extract.

3. Results and discussion

The POD from different vegetables sources (radish, cabbage, green pepper and turnip) was purified in a few steps, including extraction, ammonium sulphate precipitation, dialysis and DEAE cellulose chromatography. A summary of the purification procedure and specific information on the degree of purification for each step is shown in Table 1.

The enzyme precipitation by ammonium sulphate improved peroxidase purification and concentrates the crude extract. The specific activity and purification-fold following precipitation with ammonium sulphate were 1.7 for radish, 1.3 for cabbage, 2.1 for green pepper and 2.05 for turnip. The increase of POD specific activity after ammonium sulphate precipitation (Table1), suggests that purification-fold was enhanced by addition of ammonium sulphate.

Table 1 Level of purification of POD from vegetable samples

Vegetable sample	Steps of purification	Volume (ml)	Protein		Enzyme			
			Conc. (mg/ml)	Total amount (mg)	Activity (unit/ml)	Specific activity (unit/mg)	Yield (%)	Purification Fold
Radish	Extract	400.0	1.304	521.7	2729.5	2092.9	100.0	1.0
	$(NH_4)_2SO_4$ ppt	100.0	1.190	119.0	4258.0	3557.0	39.0	1.7
	Dialysis	35.0	0.1900	6.800	3041.0	15654.0	25.0	4.4
	DEAE	10.0	0.002	0.02	319.4	159677.0	3.0	10.2
Cabbage	Extract	400.0	1.116	446.3	427.4	383.0	100.0	1.0
	$(NH_4)_2SO_4$ ppt	100.0	2.152	215.2	1071.0	497.9	62.7	1.3
	Dialysis	35.0	0.3750	13.13	952.4	2539.0	31.1	5.1
	DEAE	10.0	0.005	0.05	80.0	16000.0	2.4	6.3
Green Pepper	Extract	400.0	2.841	1136.0	3350.0	1179.0	100.0	1.0
	$(NH_4)_2SO_4$ ppt	100.0	2.121	212.1	5253.0	2476.0	39.2	2.1
	Dialysis	35.0	0.4714	16.50	4203.0	8915.0	28.0	3.6
	DEAE	10.0	0.012	0.12	823.8	68651.0	5.6	7.7
Turnip	Extract	400.0	9.028	3610	18356.0	2033.0	100.0	1.0
	$(NH_4)_2SO_4$ ppt	100.0	8.755	875.5	36492.0	4168.0	49.7	2.05
	Dialysis	35.0	1.587	55.54	21165.0	13338.0	20.3	3.2
	DEAE	10.0	0.023	0.23	3037.0	132055.0	4.1	9.9

The precipitated enzyme of the vegetables was dialyzed by dialysis membrane for desalting the ammonium sulphate. The results indicate that the purification enhanced by dialysis process as it shown in purification fold.

The enzyme was further purified by DEAE cellulose chromatography which is the most often used cellulosic anion exchanger, for this purpose a chromatographic column was used. Degree of purification was as following, for radish was 10.2, cabbage was 6.3, green pepper was 7.7 and turnip was 9.9 folds with DEAE cellulose chromatography. The results show that the radish was the best source of the POD among the studied vegetables.

The specific activity of the purified enzyme was about 76 times more than the crude enzyme with radish, 32 times more than the crude enzyme with cabbage, 58 times more than the crude enzyme with green pepper and 65 times more than the crude enzyme with turnip in the final step of purification.

The influence of 22.5 mM hydrogen peroxide on enzyme activity is demonstrated in Fig.1 . As can be seen the enzyme activity were measured at different volumes of hydrogen peroxide. The enzyme gave maximum activity when the added volume of hydrogen peroxide was 0.8ml

for radish sample, 1ml for turnip sample, 1.1ml for pepper sample and 0.9ml for cabbage sample. The highest activities for POD were obtained for all samples in the range 0.8-1.1ml plant samples.

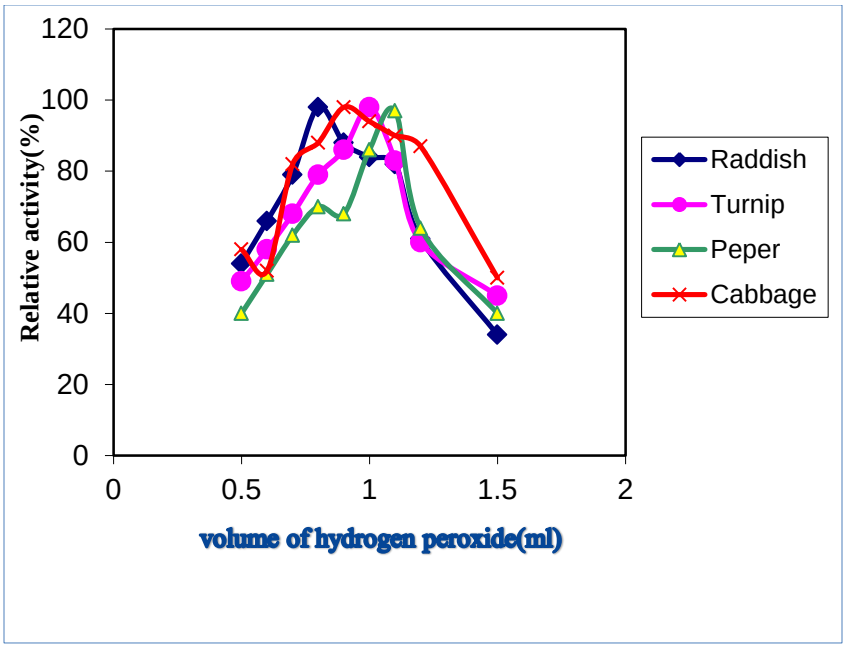

Fig. 1: Effect of hydrogen peroxide volumes on POD activity

The effect of guaiacol volumes on enzyme activity is shown in Fig. 2. The POD gave best activity in the range 1.2ml to 1.5ml for different selected vegetables. For radish and turnip highest activity was obtained when 1.2ml of guaiacol was added while for pepper extract 1.3ml of guaiacol should be added for getting maximum enzyme activity and 1.5ml of guaiacol for cabbage gave highest activity of the enzyme**.**

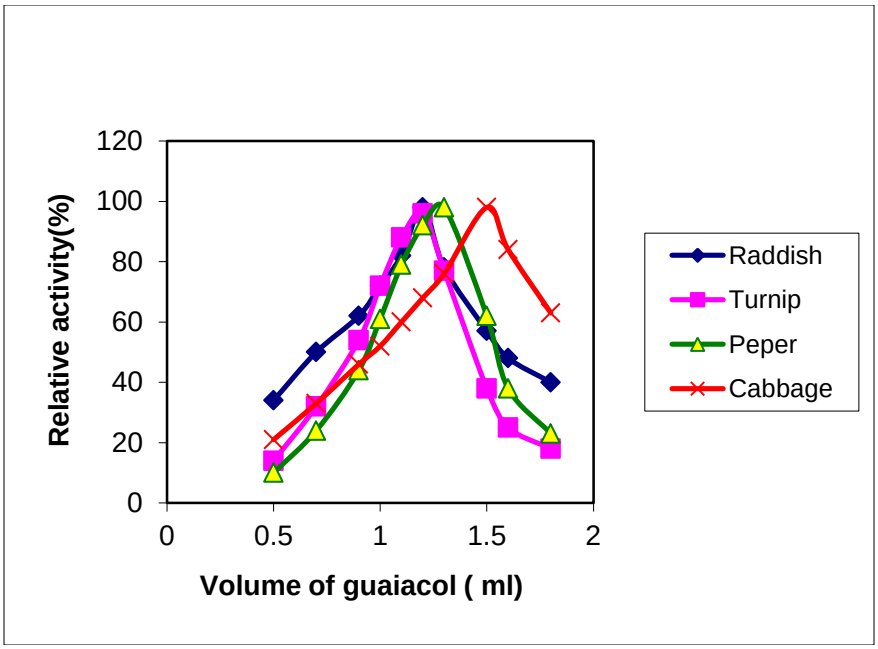

Fig. 2: Effect of guaiacol volumes on POD activity

The impact of pH on POD activity was measured by varying the pH level (Fig.3). It was found that the enzyme had the highest activity at pH 6.5 for turnip, pepper and cabbage while it shows maximum activity at pH 7.0 for radish POD. The enzyme shows good activity in a wide range but under acidic conditions, loss of activity is considered to be due mainly to heme splitting from the enzyme, whilst at neutrality, the heme bond to the protein is stable and activity is lost through chemical changes to the heme through denaturation [24].

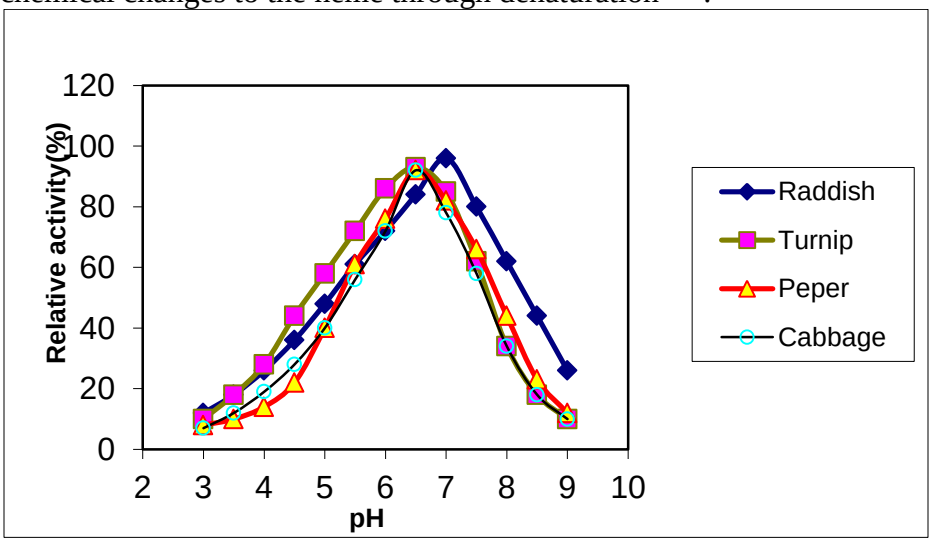

Fig. 3: Effect of pH on POD activity

The effect of the reaction temperature on the enzyme activity was investigated under optimal pH from 10 to 90 °c . The extracted enzyme of all vegetable sources has maximum activity at 30 °c. The activity decreases as temperature increases this change may be due to the splitting of covalently bound neutral carbohydrates, but this effect had not yet proven [25].

The stability of the extracted POD was evaluated after incubating the enzyme with different temperatures. The results in Fig.4 demonstrated that POD was stable over a wide range of temperature values.

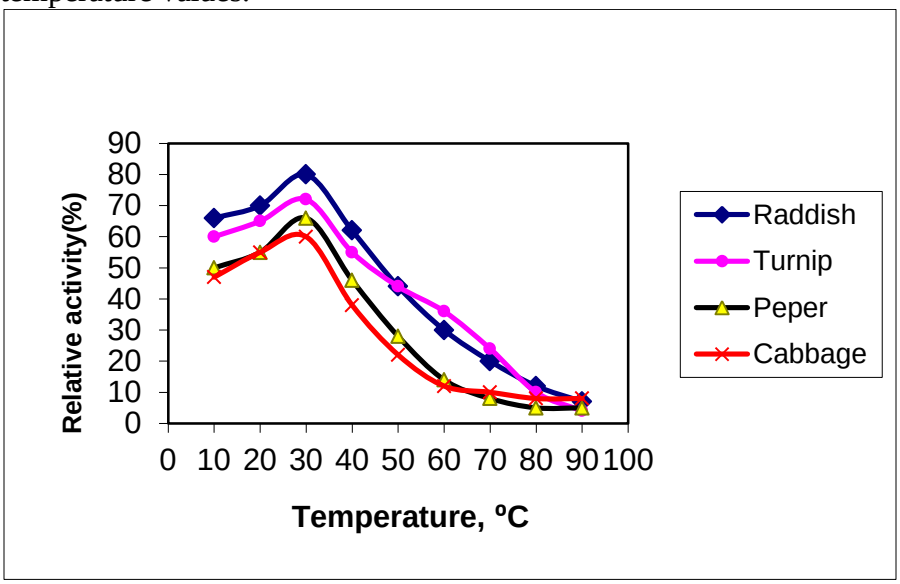

Fig. 4: Effect of temperature on POD activity

Antimicrobial activity was not exhibited by the crude aqueous extracts of radish and green pepper while the crude aqueous extract of trunip inhibited *S.areus* and E.coli , cabbage exhibited antimicrobial activity against *E.coli*, (Table.2). The antimicrobial activity of tetracycline standard also showed inhibitory effect on E.coli, Bacillus subttilis and S.aureus respectively.

The lack of antimicrobial activity in aqueous extracts may be due to the absence of antimicrobial components in these extracts, due to the interference of pigments and phenolics with the antimicrobial activity of these extracts [(26)].

Table 2 Antimicrobial activity of selected vegetables.

Extracts	E.coli	Bacillus subtilis	S.aureus
Tetracycine	+	+	+
Radish Aqueous	-	-	-
Trunip Aqueous	+	-	+
Cabbage Aqueous	+	-	-
Green pepper Aqueous	-	-	-

+ sign indicates activity and – sign indicates no activity.

Conclusions

Peroxidase enzyme from selected vegetables was extracted, partially purified and characterized by determination of some biochemical properties .It was showed that peroxidase, partially purified from various vegetables at only two steps, ammonium sulphate and DEAE cellulose chromatography. According to the result, radish was found that has higher POD activity among the other studied vegetables.

H_2O_2 was used as constant substrate for POD, guaiacol was used as changeable substrate at the kinetic studies, the result has shown, that is the, POD has higher substrate specificity for guaiacol substrate. The optimum pH was between (6.5-7.0) among vegetable sources, the extracted enzyme of all plant sources has shown maximum activity at 30c°. The crude aqueous extract of these selected vegetables hasn't showed antimicrobial activity that may be due to the interference of phenolic and pigmentation with antimicrobial activity of these extracts.

The present study showed that this enzymes is an interesting candidate for further studies such as in chemical diagnosis, also, it can be used for the application such as synthesis of various aromatics compound, removal of phenolic from waste waters and removal of peroxide from foodstuffs, beverages and industrial wastes.

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