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APPLICATION OF EXTRACTED PEROXIDASE ENZYME FROM TURNIP ROOTS (*BRASSICA NAPUS*) IN CLINICAL DIAGNOSTIC KIT

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ABSTRACT

The peroxidase enzyme was extracted by juicing the turnip roots followed by centrifugation at 12000 rpm. Partial purification of the enzyme was carried out by precipitating of crude enzyme solution with solid $(NH_4)_2SO_4$ and dialyzed against phosphate buffer saline (PBS) then fractionated by gel filtration on Sephadex G-75 column. The fractionation pattern of the pre-dialyzed enzyme illustrated that two peaks of protein content (absorbance at 280 nm) were present. The 1st peak contained most of the peroxidase activity. The 2nd peak contained other proteins. The enzyme activity showed 2.5 Ku/1ml when guaicol was used as a substrate. For glucose clinical diagnostic kit, two reagents were prepared: The 1st was commercial glucose kit as control. The 2nd was completely prepared using the extracted peroxidase (100 units/100ml reagent). The stability of the prepared kits was in agreement with those of commercial kit. They were stable up to one year when tested corresponding to the reference range of glucose; Normal: 74-102 mg/dl and Pathogenic: 235-325mg/dl.

Keywords: Peroxidase, Turnip, Extraction, Purification, and clinical diagnostic kit

INTRODUCTION

Peroxidases represent a group of enzymes widely distributed in the plant kingdom and has been extracted from several plants including horseradish (*Armoracia rusticana*), peach (*Prunus persica*), yam (*Alocasia macrorhiza*), manioc (*Manihot utilissima*), artichoke (*Cynara scolymus*), sweet potato (*Ipomoea batatas*), zucchini (*Cucurbita pepo*) and others. In this sense, another potentially interesting source of peroxidase is turnip (*Brassica napus*) [1, 2, 3]. Peroxidases are a group of haem – containing enzymes that present wide substrate specificity. The reaction catalyzed is the reduction of peroxides at the expense of electron donating substrates. This characteristic makes peroxidases useful in a number of industrial and analytical applications [4]. They can be used for the treatment of wastewater containing phenols and aromatic amines, in bio-bleaching processes, in lignin degradation, in fuel and chemical production from wood pulp, and in the biotransformation of organic compounds [1, 4, 5]. Peroxidase enters in the production of Glucose, Uric acid, Triglycerides and Cholesterol diagnostic kits. Immunoassay techniques as ELISA with labeling of reactants by horseradish peroxidase and applying of its catalytic properties are proposed [6].

METHODS

Extraction and partial purification of peroxidase from turnip roots:

Peroxidase was partial purified from 10 kg of turnip roots as mentioned before [2,3,7,8]. The roots were washed, sliced, and homogenized with 1 liter of distilled H_2O in a blender. Homogenates were then filtered through four thicknesses of cheesecloth then centrifuged at 12000 rpm for 20

minutes. Solid ammonium sulfate $(NH_4)_2 SO_4$ was added to crude enzyme solution to reach 65% saturation through stirring slowly in an ice bath. After standing at 4°C for one hour , the precipitate was removed by centrifugation at 12000 rpm for 30 minutes at 4°C. Pellets were re-suspended in a small volume of phosphate buffer saline (PBS), pH 7.5 containing 10 mM $CaCl_2$, and dialyzed overnight at 4°C against the same buffer. Samples were then taken to determine enzymatic activity. The enzyme was purified further through Sephadex G-75 gel filtration column. Sephadex G-75; 5 g was suspended in excess of PBS buffer containing 10 mM $CaCl_2$ in order to be swelled. The slurry was poured carefully into (1.5 × 30cm) column. The bed height was adjusted to 30 cm by settling the gel beads. The column was then washed and equilibrated with PBS buffer at a flow rate of 36 ml/ hr using a peristaltic pump at 4°C. The dialyzed peroxidase was applied to the Sephadex G-75 column with the aid of an adaptor. The enzyme was eluted with PBS buffer, pH 7.5 containing 10 mM $CaCl_2$ at a flow rate of 36 ml/hour. Fractions were collected at 4°C after which the absorbance at 280 nm (an indication for protein content) and the enzyme activity were determined. Active fractions were collected and re-precipitated on ice with solid $(NH_4)_2 SO_4$.

Peroxidase activity and total protein determinations:

Peroxidase activity was determined in triplicate by measurement of the absorbance at 470 nm of tetraguaiacol. The reaction mixture was 450 µl 0.1M Acetate buffer, 750µl 0.04M guaicol, 200 µl , 0.02M H_2O_2 and 100 µl sample as described [9]. Unit of enzyme is that amount of enzyme which catalyses the conversion of one micromole of hydrogen peroxide per minute at 25°C. Total protein concentration was determined in triplicate, using bovine serum albumin as standard, according to Bradford method [10].

Protein electrophoresis (SDS – PAGE):

The partial purified peroxidase was used for protein electrophoresis. For SDS-PAGE; 100µg from each sample were boiled in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol then separated in a 12% gel according to Laemmli method [11]. The proteins were stained with coomassie brilliant blue R-250**.**

Glucose level assay

Enzymatic determination of glucose:

In order to determine the glucose concentration in human serum, two reagents were prepared: The 1st was commercial clinical glucose diagnostic kit. The 2nd was completely prepared in our lab using the extracted peroxidase (100 units/100ml reagent) as described before [12].

Assay Principle

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The formed hydrogen peroxide reacts under catalysis of peroxidase with phenol and 4-aminoantipyrine to form a red violet quinoneimine dye as indicator [12].

$$\text{Glucose} + 2H_2O + O_2 \xrightarrow{GOD} \text{Gluconic acid} + H_2O_2$$

$$2\,H_2O_2 + \text{Phenol} + \text{4-amino-antipyrine} \xrightarrow{peroxidase} 4H_2O + \text{Quinoneimine}$$

Procedure

Ten **µ**l specimen was added to 1.0 ml Reagent mixture (Phosphate Buffer; 100 mmol/L, Phenol; 4.0 mmol/L, 4-amino-antipyrine; 1.0 mmol/L, Glucose oxidase ; > 20 KU /L, Peroxidase ; > 2.0 KU/L and Sodium Azide 8 mmol/L.) Glucose standard (St) concentration was 100 mg/dl, 5.55 mmol/L .The tubes were mixed and incubated for 10 minutes at 37°C or 20 minutes at 15 -25°C. The absorbance of specimen (A specimen) and standard (A standard) against reagent blank within 30 minutes at 550 nm was measured.

$$\text{Glucose concentration (mg/dl)} = \frac{\text{Glucose standard conc. x (A) specimen}}{\text{(A) Standard}}$$

RESULTS AND DISCUSSION

Extraction and partial purification of peroxidase from turnip roots

The fractionation pattern of the pre-dialyzed enzyme sample (3ml each) using Sephadex G-75 column is shown in (Figure1). The pattern illustrated that two peaks of protein content (absorbance at 280 nm) were present. The larger peak (fractions 10–25) contained most of the peroxidase activity, with a maximum activity of 2.5k units/ml. The smaller protein peak (fractions 26-40) contained proteins other than peroxidase. SDS – PAGE was carried out to check the presence and purity of the peroxidase enzyme sample. About 100 µg protein sample from enzyme after dialysis, and enzyme after Sephadex G-75 column was applied separately to 12% SDS PAGE. Sample after dialysis showed several protein bands more than that after gel filtration but still it needs more purification. Previously, it was proved that; the molecular weight of peroxidase is located between 38 – 45KDa [4, 7,13,14,15]. The results showed that peroxidase is located in this region and corresponds to a molecular weight about 40 KDa (Figure 2) as compared to the molecular weight marker (200 – 19 KDa). The results showed that; 10 Kilogram turnip roots gave 5 liter juice contained about 80 K units. After purification, it gave about 50 kilo units. This quantity satisfies for manufacturing of 50 liters Glucose kit reagents (500 kits/ 100Test).

Glucose level assay

Oxidation of glucose present in the peripheral blood represents the major source of cellular energy in the body. Dietary glucose is stored in the liver in the form of glycogen or converted to fatty acids and stored in the adipose tissues. The accurate estimation of glucose is important in the diagnosis and management of hyperglycemia and hypoglycemia. The most frequent cause of hyperglycemia is diabetes mellitus resulting from a deficiency in insulin secretion or action. Hypoglycemia may be the result of an insulinoma, insulin administration, and inborn error of carbohydrate metabolism or fasting. The concentration of glucose in the blood is controlled within narrow limits by many hormones, the most important of which are produced by the pancreas. The mean normal blood glucose level in humans is about 4 mM (4 mmol/L or 72 mg/dL, i.e. milligrams/deciliter); however, this level fluctuates throughout the day [16, 17,18,19,20].

In order to check the applicability of peroxidase, the enzymatic determination of a serum metabolite such as glucose was performed. In these reactions the enzyme must be present in saturating amounts in order to be sure that H_2O_2 produced in the test is converted into a colored substance [4]. The optimal concentration of peroxidase, which gave a linear response with time, was 100 units/100 ml reagents. The present study shows that, in spite of peroxidase have not been completely purified, it gave good stability in glucose kit for one year as demonstrated in (Table 1 and Fig.3). The stability of the prepared kits was in agreement with those of commercial kits. They were stable up to one year at 37 ◦C when be tested corresponding to the reference range of glucose; Normal: 74-102 mg/dl and Pathogenic: 235-325mg/dl.

CONCLUSION

In general, these results will promote using the turnip roots as alternative source of peroxidase in clinical diagnostic kits in which H_2O_2 is generated and then determined using peroxidase as glucose, uric acid, triglycerides and cholesterol or in immunoassay reagents as Western blot, Dot Blot and ELISA [4, 21,22,23].

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Figure (1): Fractionation pattern for peroxidase extracted from turnip using sephadex G-75 gel filtration column.

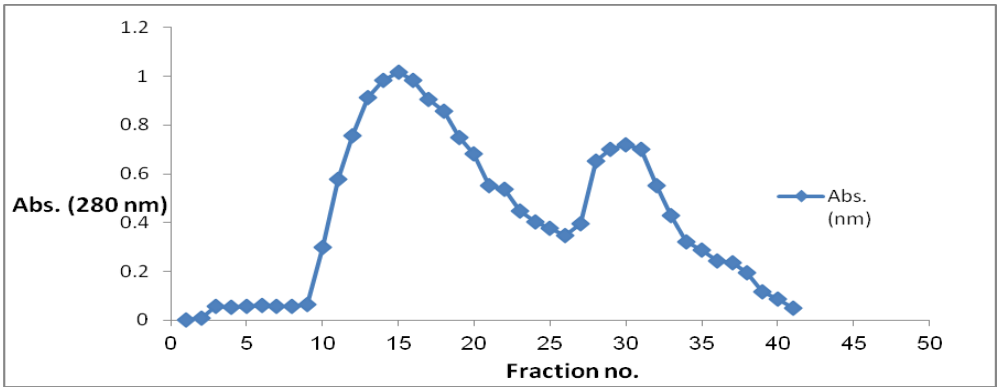

Figure (2): SDS – polyacrylamide gel electrophersis of proteins containing peroxidase extracted from turnip (M) SDS molecular weight marker (10-225 K Dalton, promega, USA), (1) sample after dialysis (2) sample after Sephadex G-75 column.

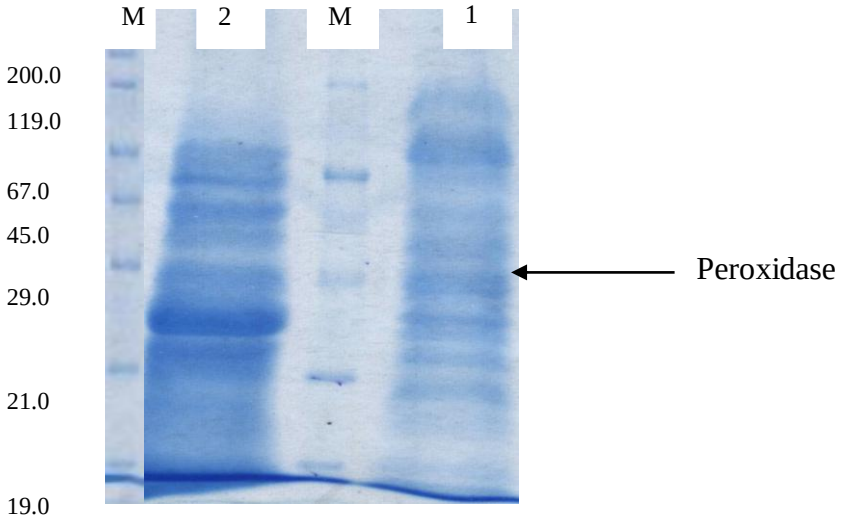

Fig (3): Following up the stability of glucose kit produced in our lab and contains the extracted peroxidase corresponding to commercial glucose kit. Series1 represents normal Lab's trial, series 2 represents normal commercial kit, series 3 represents pathogenic Lab's trial and series 4 represents pathogenic commercial kit.

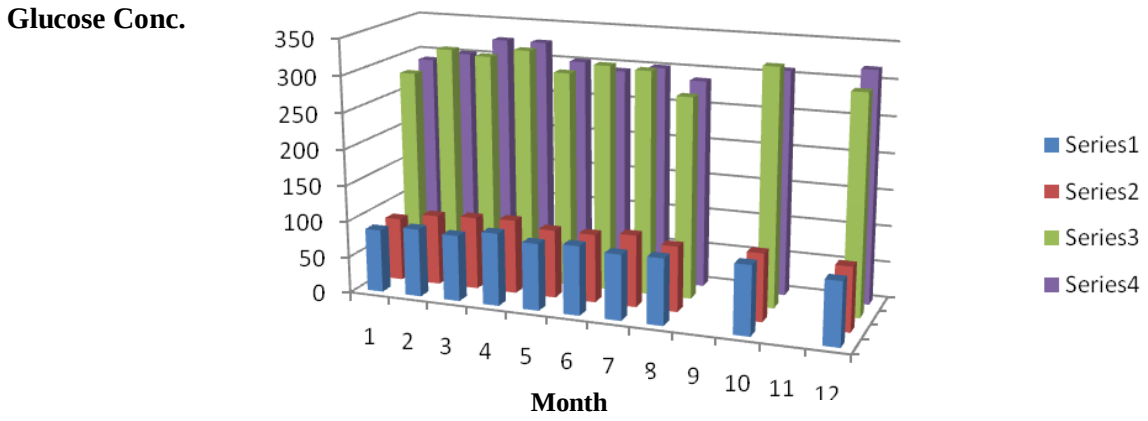

Table 1: Following up the stability of glucose kit produced in our lab and contains the extracted peroxidase corresponding to commercial glucose kit

Month	Normal (mg/dl)		Pathogenic (mg/dl)	
	Lab's trial	Commercial kit	Lab's trial	Commercial kit
1	87	87	281	290
2	94	97	318	302
3	91	100	312	325
4	100	102	324	325
5	92	94	297	302
6	95	94	311	293
7	90	99	308	301
8	91	90	277	287
10	95	93	327	309
12	87	88	300	318