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Isolatin and characterization of Peroxidase from Breast Cancer (Part II)

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ABSTRACT

The research was concerned with isolation and characterization of peroxidase from breast cancer tissues using different biochemical techniques. Two proteinous components had been isolated by gel filtration chromatography from the precipitate produced by ammonium sulphate saturation. It was found that only the second peak had peroxidase activity.

The apparent molecular weight of the isolated peroxidase using gel filtration chromatography and SDS–electrophoresis was determined and found to be (39800) Dalton.

Finally, the research specifies the optimum conditions for peroxidase activity. Maximum activity was obtained using (20) mM of guaiacol as a substrate for the enzyme, sodium phosphate (150 mM) as a buffer at pH (6) for (5) minutes at (40) °C. Using linweaver–burk plot, it was found that maximum velocity (Vmax) and Michaelis constant (km) had the values of (135.37) µmol/ min and (2.56) mM respectively. The effect of some chemical compounds and drugs on the peroxidase activity was also studied. It was found that manganase chloride ($MnCl_2$) showed a competitive inhibition on the activity of the enzyme at a concentration of (10) mmol.

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*This work is taken from her Ph. D thesis

الملخص

تضمن البحث عزل وتوصيف انزيم البيروكسيديز من انسجة سـرطان الثـدي لإحـدى المـصابات باستخدام بعض التقنيات الحياتية المختلفة حيث تم فصل حزمتين رئيسيتين من البروتينـات بتقنيـة الترشـيح الهلامي للراسب البروتيني الناتج من الترسيب بكبريتات الامونيوم واظهرت الحزمة الثانية فقط فعالية لأنزيم البيروكسيديز . وقدر الوزن الجزيئي لأنزيم البيروكسيديز باستخدام تقنيتـي الترشـيح الهلامـي والهجـرة الكهربائية وكان بحدود (39800) دالتون.

كذلك تضمن البحث ايجاد الظروف المثالية لفعاليةأنزيم البيروكسيديز، وكانت كالاتي: استخدام (20) ملي مولار من مادة كويكول (Guaiacol) كمادة اساس و(150) ملي مولار من محلول الفوسفات المنظم عند اس هيدروجيني (6) ودرجة حرارة (40) م بفترة زمنية مقدارها (5) دقائق. وباستخدام رسم لايـن ويفـر–برك تم ايجاد قيمـة الـسرعة القـصوى (Vmax) وثابـت مكـيلس (Km) وكانتـا مـساوية لـ (min 135.37µmol/) و (2.56 mM) على التوالي. أخيراً تم دراسة تأثير بعض المركبات الكيميائيـة والادويـة على فعالية أنزيم البيروكسيديز،وبينت النتائج بأن كلوريد المنغنيز ($MnCl_2$) مثبط تنافسي لفعاليـة الانـزيم بتركيز (10) ملي مولار.

INTRODUCTION

Peroxidase (E.C. 1.11.1.7) has been widely used as an important component of reagents for clinical diagnosis and various laboratory experiments (Kim and Yoo, 1996). The presence of peroxidase in most cell organelles suggests the enzyme to be intracellular, but it may also be an extracellular enzyme, considering its occurrence in the cell wall and its role in ligniuprotein complex formation (Huystee and Lobazewski, 1982).

The general principles of peroxidase action is catalyzing the oxidation by peroxide of a wide variety of substances many of which have strong absorption bands themselves or the oxidation products of which absorb strongly (Chance and Machly, 1954).

$$ROOH + AH_2 \xrightarrow{\text{Peroxidase}} H_2O + A + ROH$$

Some reports have shown that the presence of peroxidase is associated with a significant improvement in outcome among breast-cancer patients, and is of similar importance to other prognostic indices such as axillary nodal status and tumor size (Shering *et al.*, 1996; Gogas *et al.*, 2001; Turken and Narin, 2003). In the preceding paper (PartI), we reported that serum peroxidase activity in breast cancer women was increased significantly (about two folds) compared to contol. This finding encourage us to provide a detailed study of peroxidase involving isolation, characterization and purification from human breast cancer tissue using different biochemical techniques.

MATERIALS AND METHODS

Patients: Patients were enrolled in the present study at the breast-examination unit in Al-Khansaa Hospital and Al-Hafith Hospital in Nineva Governorate.

Collection of Blood Samples: Venous blood samples (5 ml) were drawn from each patient then transferred immediately to a clean dry plain tube. After removing the needle, the blood was allowed to clot for at least (10-15) min. at room temperature and then centrifuged for (10) min. at (4000 xg). Serum was removed for the measurement of peroxidase activity**.**

Determination of Peroxidase activity in Serum:

Peroxidase activity in serum was assayed colorimetrically at 470 nm which was applied for determining peroxidase activity from human placenta (Nelson and Kulkarni, 1990). The reaction mixture contained:

1 ml (0.1 M) of sodium phosphate buffer, pH = 7.2, 1 ml (13 mM) of Guaiacol and 1 ml (0.3 mM) of H_2O_2. The reaction was initiated by the addition of 50 µL serum to the working solution, and all the assays were performed at 37 °C in a water bath. One unit of peroxidase activity (U) represents the amount of enzyme catalyzing the oxidation of (1) µmole of guaiacol in (1) min.

Partial Purification and Characterization of Peroxidase:

Fresh human breast were obtained from Al-Salam General Hospital. The breast were placed on ice immediately after surgery and kept at (0) °C until used. Soft breast tissues 97 gm were homogenized in a meat grinder. The ground tissues were suspended in(1:3) volumes (w/v) of distilled water and stirred for (1) hr. at (4) °C. The suspension was then homogenized in a blender for (30) second at (4) °C. The homogenized material was refrigerated and then centrifuged at (4000 xg) for (30) minutes, and the supernatant solution was processed for the next step (Nelson and Kulkarni, 1990). Protein concentration in the extract was estimated by modified Lowry (Schacterle and Pollak, 1973) using bovine serum albumin (BSA) as a standard with extinction coefficient of 0.67 (Holm and Peck, 1988).

Ammonium Sulfate Precipitation:

Protein as an enzyme source was precipitated using ammonium sulfate saturation (Dixon and Webb, 1961). The supernatant was brought to 60% saturation with ammonium sulfate by slowly adding solid ammonium sulfate. The addition of ammonium sulfate was gradual, so that a small amount was added and allowed to dissolve before making further additions. The mixture was stirred electrically at (4) °C for (60) min., then left overnight in the refrigerator. The precipitate formed was then separated by centrifugation at (6000 xg) for (20) minutes. Protein concentration was estimated and peroxidase activity was determined.

Dialysis:

A dialysis sac (M.Wt. cut off less than 0000 Dalton) containing peroxidase preparation was placed in a large volume of (0.1 M) ammonium bicarbonate. The solution was stirred with a magnetic stirrer overnight at (4) °C. The solution was changed twice during dialysis (Robyt and White, 1987). After dialysis, the solution containing peroxidase preparation was lyophilized and the resulting powder was stored in deep freeze for the next step.

Gel Filtration Chromatography:

A concentrated sample of (2) ml of the proteinous material from the previous step was applied to a column (2.2 × 50 cm) containing sephadex G-75 to (45) cm height. Elution of the proteinous materials was carried out at a flow rate of (60) ml/hour using distilled water as eluant. The proteinous compound and peroxidase activity in each fraction collected were detected by following the absorbance at wavelength (280) and (470) nm. Each peak was combined separately from the plot of absorbance versus elution volumes. A powdered

proteinous compound from each peak was obtained by lyophilization, then kept in a freezer in a tight sample tube for further experiments.

Electrophoresis:

A sample from each peak after gel filtration was applied on SDS-PAGE using disc electrophoresis unit P.A.G.E. quick fit instrumentation (Robyt and White, 1987).

RESULTS AND DISCUSSION

Enzyme Isolation:

Isolation and purification of biological molecules (proteins, carbohydrate, nucleic acid and lipid) can be under special laboratory techniques of cell lysis, tissue homogenization, filtration, centrifugation, chromatography, and salt or organic solvent precipitation and concentration (Robyt and White, 1987). As with many other biological materials, extreme conditions must be avoided when attempting to isolate proteins, and physical rather than chemical methods are employed. Generally speaking a high protein concentration, low temperature and pH close to neutrality are best, otherwise denaturation occurs (Plummer, 1978).

Precipitation of the Protein:

The most commonly used salt is ammonium sulfate, which has a high water solubility (Robyte and White, 1987). The results predicted that a 60% saturation with ammonium sulfate to crude preparation (after homogenized the soft breast tissues and centrifuged) produced maximum protein precipitate. The precipitate was dialyzed to remove the small molecular compounds.

Gel Filtration:

This technique was applied to separate the proteinous materials, which were obtained by ammonium sulfate precipitation method and dialysis for crude preparation from soft breast cancer tissue (Nelson and Kulkarni, 1990). The results of elution shown in Figure (1) indicated that there were mainly two proteinous compounds, A and B. Peak B was obtained with high peroxidase activity (19.06 μ mole/min.), while peak A with very low peroxidase activity (0.0977 μ mole/min.) which was neglected.

The specific activity of enzyme was higher in peak B (8.54) fold than the activity in crude extract as shown in Table (1).

Table 1: Partial purification of peroxidase from breast cancer tissue

Fraction or step	Total protein (mg/ml)	Total activity (U/min)	S.P. Activity (U/mg)	Purification fold	Yield of original activity
Initial extract	4.2	133.90	31.88	1	100
$(NH_4)_2SO_4$	3.3	215.05	65.16	2.04	160.58
Dialysis	2.8	260.49	93.03	2.918	194.5
G-75 Sephadex Peak A	0.05	0.0977	1.95	0.0612	0.072
Peak B	0.07	19.060	272.29	8.54	14.23

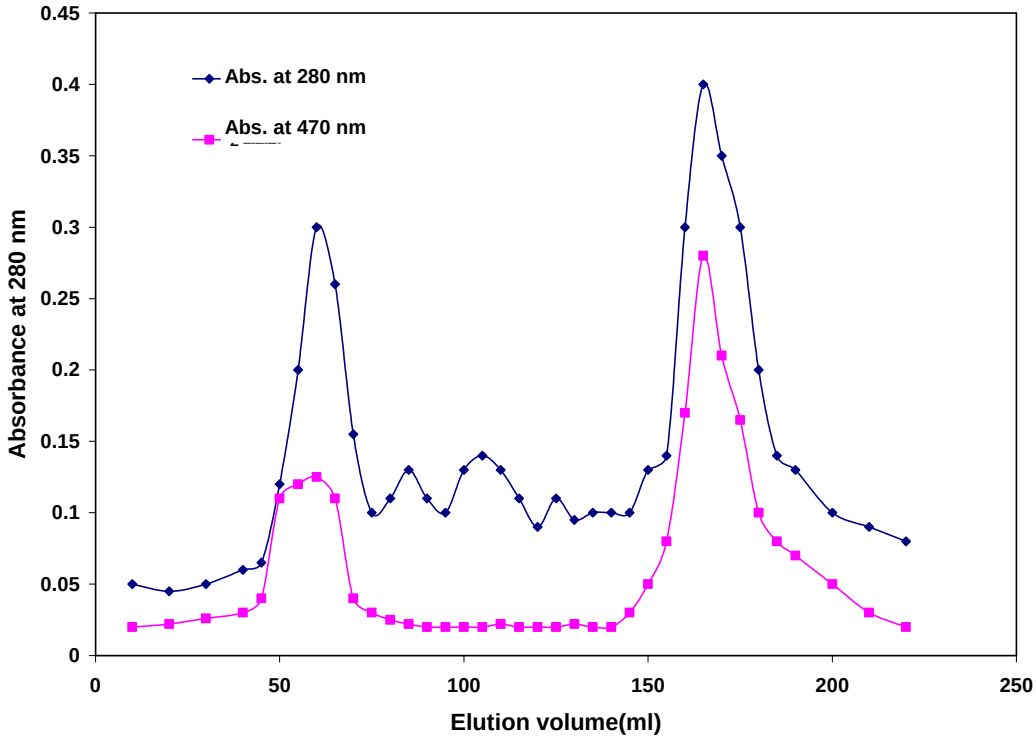

Fig.1: Elution profile of peroxidase activity on Sephadex G-75. The dimension of the column are (2.2 × 45 cm) each fraction is (3 ml) at a flow rate of 1 ml/min.

Molecular Weight Determination:

The molecular weight of peak B as a source of peroxidase was determined by the elution volume from a Sephadex G-75 column (2.2 × 45) cm calibrated with known molecular weight proteins that as listed in Table (2).

Then from the plot of logarithmic molecular weight of each material indicated in Table (2). Versus the elution volumes giving a straight line .

Table 2: Elution volumes of known molecular weight materials on Sephadex G-75

Materials	Molecular weight (Dalton)	Elution volume (ml)
Blue dextran	2000000	60
Bovine serum albumin	67000	78
Amylase	58000	117
Egg albumin	45000	147
Trptophan	204	228
Peak B	39800	169

The molecular weight of unknown proteinous compound separated by the same column chromatography could be determined from the standard curve. The comparative molecular weight of peak B as a source of peroxidase enzyme is approximately equal to (39800) Dalton. This result is highly agrees with previous results published by other

investigators who found that the molecular weight of peroxidase purified from different sources was in the range of (39,000 ± 2000) Dalton (Nelson and Kulkami, 1990; Ahmad and Hamody, 2001).

Molecular Weight Determination by SDS-PAGE Electrophoresis:

Electrophoresis on polyacrylamide gel in the detergent sodium dodecyl sulfate Na Dod $SO_4^-[CH_3(CH_2)_{10}CH_2OSO_3^-]Na^+$, abbreviated SDS, is a rapid and often employed technique for the determination of the molecular weight of proteins (Voet and Voet, 1990).

In this study peroxidase enzyme exhibited only one single band, using SDS-electrophoresis and the molecular weight of peroxidase was determined using known molecular weight compounds and found to be in the range of (39800) Dalton as shown in Figures (2). This result approximately similar to that obtained from gel filtration chromatography. This finding agree well with previous results where it was reported that the molecular weight of peroxidase on SDS-PAGE electrophoresis is (40000) Dalton (Nelson and Kulkarri, 1990; Ahmad and Mohammad, 2001).

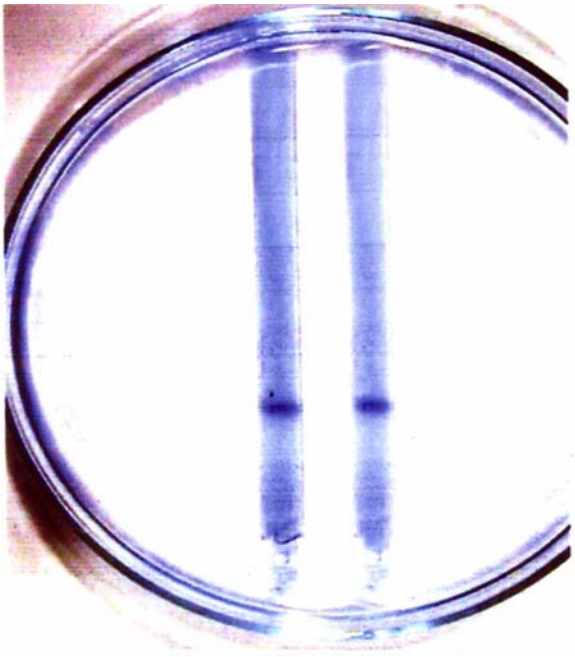

Fig. 2: SDS-Polyacrylamide gel electrophoresis of partially purified peroxidase from breast cancer tissue (left) and standard peroxidase from Sigma (right).

Optimum Conditions for Peroxidase Activity:

To develop assay conditions where peroxidase shows a maximum activity, a series of experiments were performed. These included changing the enzyme concentration, the incubation time, the incubation temperature, buffer concentration, the pH of the assay conditions and the substrate concentration.

Effect of Enzyme Concentration on Peroxidase Activity:

It is important to establish that the activity varies linearly with enzyme concentration. The activity of enzyme was measured in the presence of different concentrations of partially purified enzyme from breast cancer tissue between (10-70) µg/ml as shown in Figure (3).

The result indicated that the enzyme activity increased linearly with increasing the concentration of the protein as a source of the enzyme. These results were in direct accord with most enzymes where the activity increases with increasing the enzyme concentration provided no inhibitors are present.

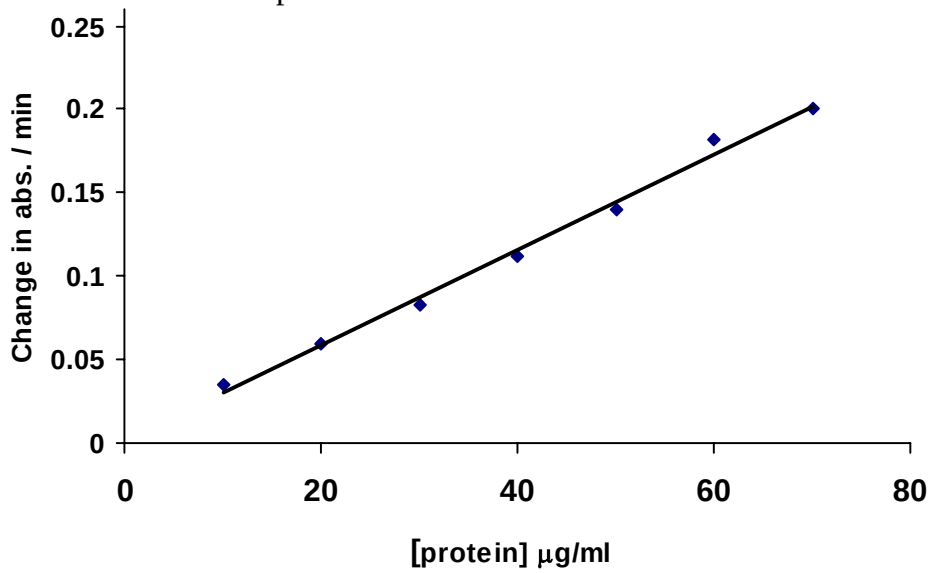

Figure 3: Effect of different enzyme concentration of peroxidase activity

Incubation Time as a Function of Enzyme Activity:

To determine the stability of peroxidase activity under assay conditions, a series of experiments were performed at different time intervals. The results indicated that maximum enzyme activity was obtained after (5) min. (Figure 4).

Effect of Temperature on Peroxidase Activity:

The role of an enzyme-catalysed reactions, like most chemical reaction, increases with temperature. This means that the initial reaction rate will rise with temperature until it becomes impossible to measure due to almost immediate inactivation. In practice, most enzymes are completely inactivated above (70 °C) (Plummer, 1978).

In this study, it has been found that as the temperature increased, there was a concave up increase in the enzyme activity until it reached a maximum value at a temperature of (40 °C) then dropped gradually until most of it was lost at (80 °C) Figure (5).

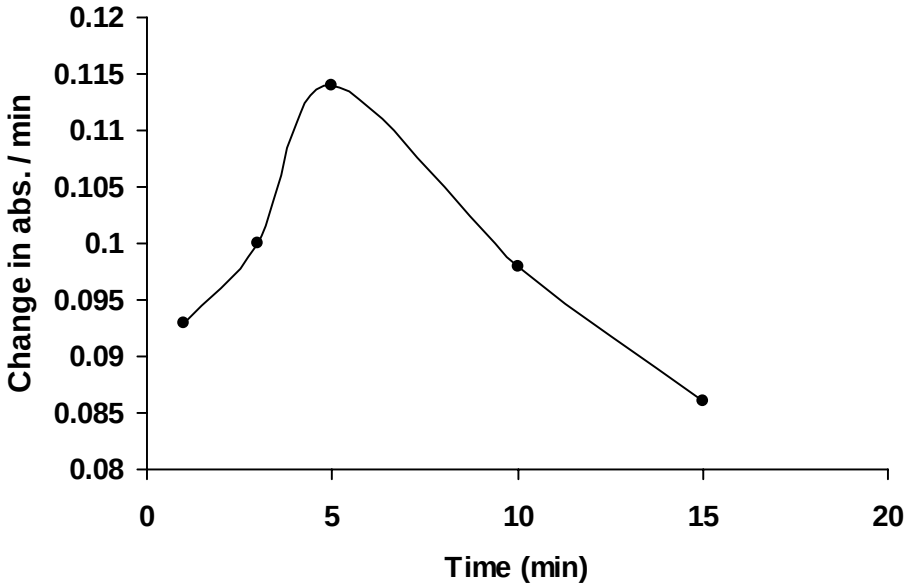

Figure 4: Effect of incubation time on peroxidase activity

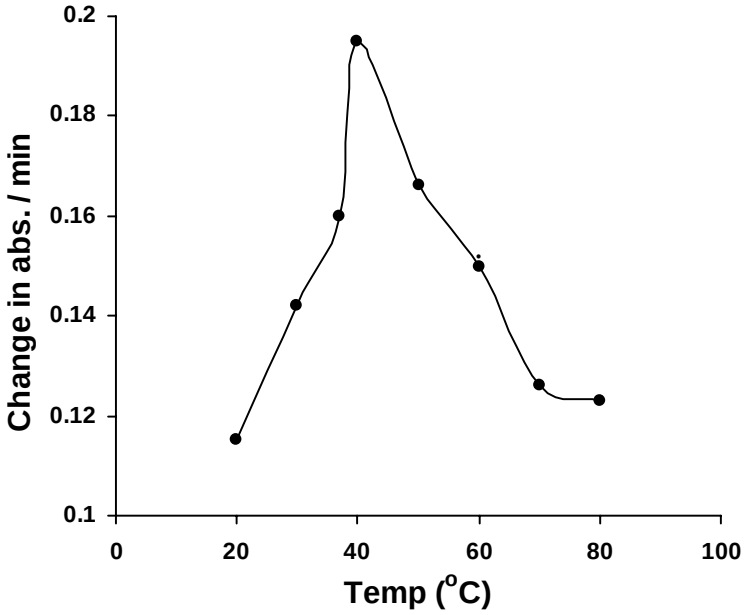

Figure 5: Effect of temperature (°C) on peroxidase activity

Effect of Buffer Concentration of Peroxidase Activity:

It is important to establish that activity varies with buffer concentration. The activity of enzyme was measured in the presence of a different concentration of buffer solution within the range (50-200) mM of sodium phosphate buffer. Maximum activity was obtained using (150) mM of sodium phosphate buffer (Figure 6).

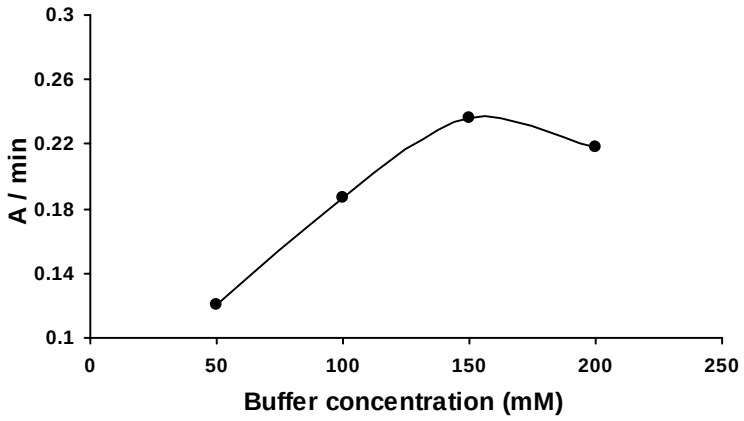

Figure 6: Effect of buffer concentration on peroxidase activity

Effect of pH on Peroxidase Activity:

The influence of pH upon the activity of peroxidase was investigated using buffer solution containing (150) mM of sodium phosphate with a pH range of (3-8). In this case the assay conditions were conducted in the same manner as described earlier except that the pH was varied as indicated in Figure (7). The optimum pH for peroxidase was found to be (6) in sodium phosphate buffer.

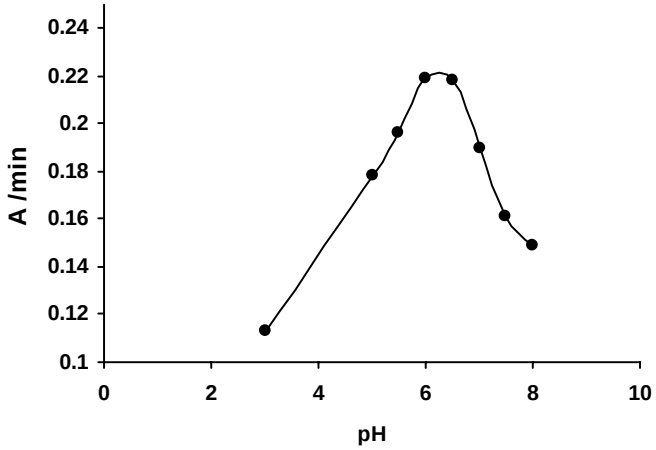

Figure 7: Effect of pH on purified peroxidase activity using (150) mM sodium phosphate buffer.

Effect of Substrate Concentration on the Enzyme Activity:

For many enzymes, the rate of catalysis (U) varies with the substrate concentration. The rate of catalysis (U) is defined as the number of moles of product formed per second at a fixed concentration of enzyme. (U) is almost linearly proportional to [S] when [S] is small. At high [S], (U) is nearly independent of [S] (Walsh, 1979).

To determine the effect of substrate concentration on the enzyme activity, a series of experiments were performed where the concentration of the substrate was varied Figure (8). Assays were conducted as described previously using guaiacol in concentration range of (5-

35) mM. Maximum activity was obtained with (20) mM of guaiacol. Further, increase in the concentration of the substrate did not alter the activity of the enzyme.

Figure 8: Effect of substrate concentration on peroxidase activity.

The Michaelis-Menten constant (Km) of the enzyme was determined from Figure (8) and found to be (2.50) mM. A similar result was obtained using a Lineweaver-Burk plot by plotting the reciprocal of the initial velocity versus the reciprocal of the substrate concentration. A linear relationship was obtained Figure (9) giving a Km value of (2.56) mM and V_{max} (135.37) μM/min.

Fig. 9 :Lineweaver-Burk plot of purified peroxidase.

Inhibition Studies of Peroxidase

The activity of an enzyme can be reversibly deceased by the noncovalent binding of inhibitors. The study of enzyme inhibitors is important because many drugs function as enzyme inhibitors (Plummer, 1978; Robyte and White, 1987). Many invastigators observed that some chemical compounds have an inhibitors effect on peroxidase activity (Nelson and Kulkarni, 1990; Greg et al., 1995; Ahmad and Hamody, 2001).

The results of adding of various compounds on the activity of the purified peroxidase are listed in Table (3).

Table 3: Effect of various chemical compounds and some drugs on the activity of the purified peroxidase from breast cancer tissue

Compound and drugs	Enzyme activity μmol/min	Y. of original activity
Standard	63.53	100
$MgCl_2$ (5 mM)	43	67.7
$MgCl_2$ (10 mM)	42.5	66.9
$MgCl_2$ (15 mM)	51.8	81.5
KCN (5 mM)	55.7	87.7
KCN (10 mM)	21.5	33.8
KCN (15 mM)	29.3	46.1
$MnCl_2$ (5 mM)	20.5	32.3
$MnCl_2$ (10 mM)	8.79	13.8
$MnCl_2$ (15 mM)	9.77	15.4
NaN_3 (5 mM)	41.05	64.6
NaN_3 (10 mM)	33.7	53.0
NaN_3 (15 mM)	29.8	46.9
NaF (5 mM)	8.84	13.9
NaF (10 mM)	34.2	53.8
NaF (15 mM)	26.87	42.3
$CaCl_2$ (5 mM)	38.6	60.8
$CaCl_2$ (10 mM)	32.74	51.5
$CaCl_2$ (15 mM)	28.34	44.6
Na_2SO_3 (5 mM)	42.03	66.2
Na_2SO_3 (10 mM)	42.33	66.6
Na_2SO_3 (15 mM)	42.51	66.9
Tamoxifen	17.10	26.9
Paracetamol	51.16	80.5
Ponstan	50.38	79.3
Aspirin	23.94	37.7
Primolut N	24	37.8
Dopo-provera	25.65	40.4
Microgenon	19.54	30.8
Neugenon	51.31	80.8

The results in Table (3) showed that the addition of many chemical compounds at different concentrations (5-15) mM and some drugs used for many breast cancer women to the assay mixture of the enzyme had an inhibitory effect on peroxidase activity. Maximum inhibition was observed when $MnCl_2$ was used at a concentration of (10 mM), NaF at a concentration of (5 mM) and Tamoxifen which was used as a chemotherapy treatment. This finding is similar to what has been reported in the literature (Nelson and Kulkarni, 1990; Zeneca, 1998; Ahmad and Hamody 2001).

In this study, maximum inhibition was obtained when $MnCl_2$ was used at a concentration of (10 mM) for a type of inhibition on the enzyme. A line Weaver-Burk plot was performed Figure (10) where (10 mM) of $MnCl_2$ as an inhibitor was used. The results showed that V_{max} remained constant while Km increased with increasing the concentration of inhibitor. This finding predicted that $MnCl_2$ acted as a competitive inhibitor. Similar observations had been reported in the literature (Nelson and Kulkarni, 1990; Greg *et al.*, 1995).

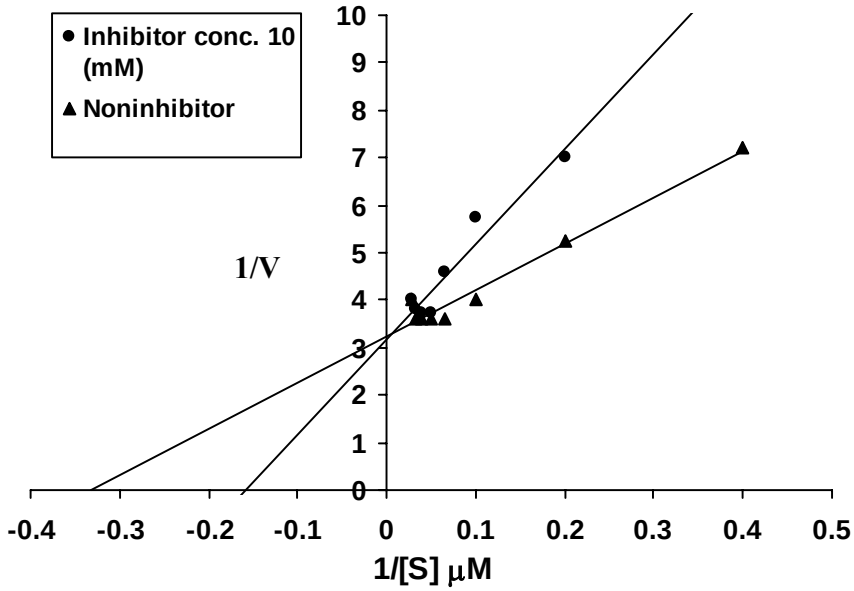

Figure (10): Lineweaver-Burk plot of peroxidase in the presence of 10 mM of $MnCl_2$.

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