DEGRADATION OF REACTIVE DYES USING IMMOBILIZED PEROXIDASE PURIFIED FROM *NIGELLA SATIVA*

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ABSTRACT

The present study was aimed to exploit the free and immobilized peroxidase from *Nigella sativa* seeds to degradation of textile dyes polluting the environment and water. The optimum conditions for extracting the enzyme from the *Nigella* seeds were determined and the highest specific activity of the enzyme was obtained 1750 units / mg protein when extracting the enzyme from the ground seeds at a ratio of 1:20 (w: v) with sodium acetate buffer at 0.2 M and pH 5.0 for 15 minutes. The enzyme was purified using two steps including the concentration by sucrose and gel filtration by using Sephadex G-150. The results shown an increase in final purification folds 2.8 time with an enzyme yield of 35%. The immobilization of peroxidase were done by entrapment method using Ca- alginate and the immobilization ratio was reached to 49%. The removal efficiency of dyes by crude enzyme (free, immobilized) and partial purified peroxidase were studied with textile dyes such as yellow, red, black and blue dyes at optimum conditions pH 5, temperature 37°C after 3 hr. Maximum removal efficiency of dyes observed with crude peroxidase and reached (76.9, 88.7, 91 and 88) % respectively. These results were close to the efficiency of the purified enzyme in removing the four dyes, while the efficiency of the crude immobilized enzyme in removing the dyes was about (70, 81, 72 and 56.4)%, respectively.

Keywords: plant, enzyme, black seed, textile dyes

عزیز وآخرون

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تفكيك الصبغات التفاعلية بواسطة انزيم البيروكسيداز المقيد والمنقى من نبات حبة البركة

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المستخلص

هدفت الدراسة الحالية الى استغلال البيروكسيداز من بذور حبة البركة بصورته الحر والمقيد في تفكيك بعض صبغات معامل الانسجة الملوثة للبيئة والمياه. حددت الظروف المثلى لاستخلاص الانزيم من بذور حبة البركة وتم الحصول على اعلى فعاليته نوعيه للانزيم 1750 وحدة / ملغم بروتين عند استخلاص الانزيم من البذور المطحونه بنسبه 1:20 (وزن : حجم) بدائى خللات الصوديوم بتركيز 0.2 مولار ورقم هيدروجيني 5.0 لمدة 15 دقيقه . نقي الانزيم باستخدام خطوتين شملت التركيز باستخدام السكروز والترشيح الهلامي باستخدام مادة السيفادكس -ج 150 ، بينت النتائج زيادة في عدد مرات التنقيه النهائيه اذا بلغت 2.8 بحصيله انزيميه وصلت 35%. قيد انزيم البيروكسيداز بوساطه طريقه الحجز باستخدام الجئات الكالسيوم حيث بلغت نسبه التقييد 49%، كما قدرت كفاءة الانزيم الخام (الحر والمقيد) والمنقى في ازاله انواع مختلفه من صبغات معامل الانسجه مثل الصبغه الصفراء والحمراء والسوداء والزرقاء عند رقم هيدروجيني 5 ودرجه حرارة 37 مئويه بعد 3 ساعات من المعامله. بينت نتائج الدراسة ان الانزيم الخام الحر هو الاكفأ في ازاله الصبغات الاربعه اذ بلغت كفاءته (76.9 و 88.7 و 88 و 91) % على التوالي. وكانت هذه النتائج مقاربه الى كفاءة الانزيم المنقى في ازاله الصبغات الاربعه ، بينما بلغت كفاءة الانزيم الخام المقيد في ازاله الصبغات حوالي (70 و 81 و 72 و 56.4) % على التوالي .

كلمات مفتاحية: نبات، انزيم، حبة البركة، صبغات الاقمشة

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INTRODUCTION

Peroxidase enzymes (donor: H_2O_2 Oxidoreductase, EC. 1.11.1.7) are widely distributed in plants, animal tissue and microorganisms. (Peroxidase was first found in the fig tree in 1936. In 1941 the enzyme was isolated and characterized from horseradish (HRP) (4)). During the years of 1942 to 1956, isolation of the enzyme was reported from various sources such as yeast, potato, beans, Japanese radish and wheat. Peroxidase became important industrially and medically, it has a great economic importance through its uses in the number of diagnostics and analytical varied because of his qualities kinetic, physical and chemical suitable for such applications and perhaps the most important number of ELISA (Enzyme-Linked Immuno Sorbent Assay) in labeling antibodies or antigens in the immune reactions by attacking these enzymes on solid surfaces as it is the specifications that qualify for that one of these properties the qualities high affinity toward the material basis (32), ease of detection effectiveness configuring outputs of color, do not need a process to measure the steps separating from substrates, high persistence during storage, low costs for preparation and purification as well as its importance in the analyses of the stigma blot assays and in pigmentation tissue. It is also used in biochemical analysis to estimate the hydrogen peroxide generated by some systems, such as the oxidation of glucose, amino acids and cholesterol .. etc. (23). Also reflected the importance of these enzymes in the processing of fruits and vegetables by evaluating the content extracts of food stuff from antioxidants such as ascorbic acid, phenols, flavonoids and tannins, which working on multiple modifications during the manufacturing process and storage. As well as the economic importance of the plant and its products (dates), the palm side-off products have many uses, moreover the issue of the exploitation of the nuclei of the surplus dates for the need to produce materials with economic value be of great benefit and add value to palm and their products (11). Enzyme immobilization can endow enzymes with some additional advantageous properties. The immobilized enzymes can be used repeatedly or continuously in a variety of reactors for the

efficient recovery of costly enzymes, and be easily separated from reactions systems for reuse, which make the work-up simple and the protein of the final product uncontaminated. Furthermore, it is reported that immobilized enzymes may exhibit higher selectivity and specificity (18). The aim of this study is the extraction, purification, immobilization and decolorization of some textile dyes by peroxidase from *Nigella sativa*.

MATERIALS AND METHODS

Plant

Plant was used through this study was locally available in a market, include Black seed (*Nigella sativa*) and used as the source of peroxidase enzyme.

Optimum conditions for peroxidase extraction

Treatment of *Nigella sativa* seeds: Two treatments of black seed (*Nigella sativa*) were used for peroxidase extraction. These treatments included soaked seeds for 24 hr. and grinded seeds, sodium phosphate buffer was used for extraction the enzyme form each type of treatments. One gram of black seed was homogenized with (1:5) (w:v) of 0.2 M sodium phosphate buffer, the mixing was done by using blender at 30 °C for 15 min. The slurry was filtrate through gauzes for removing any cell debris that remains in the preparation then centrifuged at 8000 rpm for 10 min. The clear supernatant obtained represented the crude extract, and was assayed for peroxidase activity, protein concentration and specific activity in each experiment.

Peroxidase activity and protein concentration

Peroxidase enzyme activity was estimated according to the method described by Silva and Koblitz (28) using guaicol as a substrate. The oxidation of guaicol was detected by measuring the absorbance increase at 470 nm after 3 min using a VIS- spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μ mol of substrate per minute. Protein concentration measured according to the method described by Bradford (6).

Type of extraction buffer

Black seed was homogenized with different types of buffers for 15 min at 30°C for peroxidase extraction. These buffers are 0.2 M

sodium acetate buffer (pH 3, 4, 5 and 6), 0.2 M sodium-phosphate buffer (pH 7) and 0.2 M Tris-base buffer (pH 8, and 9). The enzyme activity, protein concentration and specific activity were estimated in each experiment.

Extraction ratio: One gram of black seed was homogenized in different volumes of 0.2 M sodium acetate buffer pH 5 for extraction the peroxidase for 15 min at 30°C. The extraction ratio were 1:5, 1:10, 1:15, 1:20, 1:25, 1:30 and 1:40 (w : v). The enzyme activity, protein concentration and specific activity were estimated in each experiment.

Extraction time: One gram of black seed was homogenized with 0.2 M sodium acetate buffer pH 5 in different time include 15, 30, and 60 min at 30 °C. The enzyme activity, protein concentration and specific activity were estimated in each experiment.

Purification of peroxidase

The peroxidase was purified from black seed using dialysis method for enzyme concentration, then followed by gel filtration.

Enzyme concentration by sucrose

The crude enzyme solution was concentrated by sucrose using dialysis tubes, then was taken to measure the enzyme activity, protein concentration and the specific activity.

Gel filtration chromatography

Preparation sephadex G150

The column was prepared and packed according to the instructions of the manufacturing company (Pharmacia-Sweden), approximately 20 g of sephadex-G150 was suspended in 500 ml distilled water at 90 °C and left for 3hr to ensure the swelling of gel beads with gentle agitation, then left in a cold place at 4°C overnight. The gel was washed twice with sodium phosphate buffer 0.2 M pH 7.0, after that, it was re-suspended in an amount of the same buffer, degassed using vacuum pump, packaged the gel gently in a glass column (21×1.6 cm) and equilibrated by the same sodium phosphate buffer.

Separation of enzyme through sephadex G-150 column: The concentrated enzyme by sucrose was passed through sephadex G-150 column, and the elution step was done by using 0.2M sodium phosphate buffer solution, pH 7.0 with flow rate 20 ml / h, 3 ml for each fraction. The protein fractions were estimated in each fraction at wave length 280 nm, then

enzyme activity was measured of these fractions and effective fractions was collected, the volume was measured, activity and protein concentration were estimated, then the volume was concentrated and distributed in tubes, freezed and then kept for subsequent experiments.

Immobilization of peroxidase

Entrapment in calcium alginate: Two ml of crude peroxidase (244 U/ml) was mixed with 5 ml of sterile sodium alginate solution (3%), then stirred gently for 10 minutes, the mixture obtained was extruded drop wise through the sterile syringe (10 ml) into 0.2M of cooled CaCl_2 solution to obtain small beads with 1 mm diameter and kept for 1 hr. The beads were washed with cooled CaCl_2 solution to remove the non-immobilized enzyme, the calcium alginate beads of enzyme were stored in 0.5% (w/v) CaCl_2 at 4 °C (30). Immobilized enzyme activity was determined according to Silva and Koblitz (28).

Immobilized peroxidase activity:

Immobilized peroxidase activity was estimated according to the method described by Silva and Koblitz (28) by using 30 mg of immobilized enzyme instead of 0.1 ml of enzyme. The oxidation of guaiacol was detected by measuring the absorbance increase at 470 nm after 3 min using a VIS-spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute.

Application of peroxidase

Dyes decolorization

Figure 1. Effect of Two Treatments of *Nigella sativa* seeds on Peroxidase Extraction using 0.2 M Phosphate Buffer pH 7 at 30°C and for 15 min

For decolorization experiments, the textile dyes; reactive black, reactive red, reactive blue and reactive yellow were obtained from Al-diwanayah textile factory and used in the present study. The reaction mixture for the degradation of dyes contain 5 ml of (30 mg/l) for each dyes and 2 ml of 244 U/ml of enzyme solution (crude and purified peroxidase) separately and 1 gm of immobilized enzyme as mentioned by Alam *et al*, (3) with some modification. The reaction mixture was incubated and shaken in a shaker incubator (120 rpm) at 37°C. Distilled water was used instead of enzyme solution and 1 gm of calcium alginate only used instead of immobilized enzyme in the control experiment. The degradation of a specific dye was calculated in different incubation times (0, 1, 2, 3 and 24) hr. The percentage of removal efficiency was calculated by the absorbance at λ max for each dye according to Zhang *et al*, (33).

RESULTS AND DISCUSSION

Optimum conditions for peroxidase extraction: Different bioprocess conditions that affect peroxidase extraction from black seed were optimized for maximum enzyme production, some of factors affect the extraction of peroxidase such as types of treatment, types of buffer, extraction ratio and extraction time etc. Hence, optimization of these conditions helps to reduce extraction cost and to obtain a high yield of peroxidase enzyme.

Treatments of *Nigella sativa* seeds

To study the effect of two treatments of peroxidase extraction, black seed was extracted by phosphate buffer at 30 °C for 15 min with two treatment included soaked seeds for 24 hr. and grinded seeds. As can be seen in the fig. (1), peroxidase extraction was found to be maximum in grinding treatment with specific activity of 1409U/mg, while the specific activity of peroxidase in soaked seed were 704.5 U/mg. Rudrappa *etal*, (24) founded the specific activity of peroxidase extracted from hairy root culture of red beet was reached to 600 U/mg protein.

Figure 2. Effect of Types of Buffers on Peroxidase Extraction from Black Seed at 30°C for 15 min

Figure 3. Effect of Extraction Ratio on Peroxidase Extraction from Black Seed at 30°C for 15 min

Type of extraction buffer

The specific activity of peroxidase was estimated after extraction using different buffers, and the results were illustrated in (Fig. 2). These results show that sodium acetate buffer (0.2 M, pH 5.0) was best extraction buffer with specific activity 1584 U/mg protein. While other buffers with different pH were given low specific activity. pH effected of enzymatic activity and stability can explained by the fact that protein structure of an enzyme molecule is influenced by the acidity and alkalinity of the solution because of the differences in ionization state of various amino acid residues (20). An appropriate buffer solution added to a protein mixture during the extraction process can help improve the stability of protein molecules as these molecules are subjected to various forces designed to isolate them for study. A buffer solution can protect the integrity of the proteins while separating them from other

integrated cell components, the pH balance of the buffer must correspond with that of the cell in vivo. To keep an extraction timely and efficient, and to avoid the need to switch buffer solutions during the process, it's wise to choose a buffer solution that can maintain protein stability during every stage of the procedure (13). There are many studies that used different buffers for peroxidase extraction from different sources, Harco with coworkers (14) pointed for extraction of peroxidase from *Nicotiana tabacum* by potassium phosphate buffer (50 mM, pH 7.5).

Figure 4. Effect of Extraction Time on Peroxidase Extraction from Black Seed at 30°C for 15 min

- A: Acetate Buffer pH 3.0
- B: Acetate Buffer pH 4.0
- C: Acetate Buffer pH 5.0
- D: Acetate Buffer pH 6.0
- E: Phosphate Buffer pH 7.0
- F: Tris Buffer pH 8.0
- G: Tris Buffer pH 8.0

Extraction Ratio

Seven extraction ratio were chosen 1:5, 1:10, 1:15, 1:20, 1:25, 1:30 and 1:40 (w : v) to determine the best extraction ratio of peroxidase by using sodium acetate (0.2 M, pH 5.0). The highest specific activity was measured for crude extract at 1:20 ratio, it was reached to 1750 U/mg protein, while other ratio were gave the following specific activities 1580, 1620, 1730, 875, 428 and 342 U/mg protein respectively (Fig.3). Nidadavolu *et al.*, (22) were found that the best extraction ratio for peroxidase from discards mushroom beds was 1:1 and 1:2, while (31) extracted the peroxidase from *Arthomyces ramosus* with 1:5 ratio .

Figure 5. Gel Filtration Chromatography for Peroxidase Purification from *Nigella sativa* by using Sephadex G150 Column (21 x 1.6) cm Equilibrated and Eluted with Phosphate Buffer (0.2 M, pH 7), in Flow Rate 20ml/hr. 3ml for each Fraction.

Extraction time

Three extraction times were chosen (15, 30 and 60) min to determine the best extraction time of peroxidase by using sodium acetate (0.2 M, pH 5.0). The highest specific activity was measured for crude extract after 15 min, it was reached to 1766 U/mg protein, compared to the lower of specific activity after 30 and 60 min 1377.3 and 1350 U/mg protein respectively (Fig. 4).

Purification of peroxidase

Peroxidase enzyme extracted from *Nigella sativa* by homogenization with acetate buffer (0.1 M, pH 5.0) then by cooling centrifuge at 8000 rpm for 10 min. The crude peroxidase was purified from the unwanted proteins and other components using concentration by sucrose, and gel filtration using (Sephadex – G150) and as follows:

Enzyme concentration by sucrose

The crude extract produced was initially subjected for concentration by sucrose, the results showed that 61.2 % of peroxidase enzyme was yielded with purification fold 2.5 as shown in (table 1). Sucrose was formerly widely used for the concentration of proteins, it almost used as an inexpensive way of precipitating and concentrating a protein extract, sucrose influencing as a nucleophilic effector (activator) on the enzyme. Bajaj and Singh (5) described that the method for the concentration the enzymes by reducing the volume of the active solution by dialyzing

against a concentrated solution of sucrose, through withdrawal of water molecules from the enzymatic solution. The other methods of concentration the enzyme by precipitated technique included ammonium sulfate, ethanol, and acetone, dialysing against 96 % of ethyl alcohol or acetone as the outside solution (provided only that the active substance is not inactivated by them), by this procedure the volume of the active substance is rapidly reduced (12).

Gel filtration chromatography

The solution of concentrated enzyme was passed through a gel filtration column Sephadex - G150 with the dimensions (21×1.6) cm, and equilibrium with 0.2 M of phosphate buffer pH 7. Results showed four peaks of protein in the eluted fractions from the column with a single peak of peroxidase activity then the active fractions were collected (Fig. 5). The result indicated presence of one peak of peroxidase activity in fractions (14-31), in this step the specific activity reached to 3875 U/mg protein with a purification fold 2.8 and yield reached to 35 % as shown in (table

1). Sessa and Anderson (25) were purified soybean peroxidase by gel filtration on Bio-Gel P-60. Khurshi (17) purified horseradish peroxidase by using $(\text{NH}_4)_2\text{SO}_4$ precipitation, dialysis then by gel filtration using sephadex-G75. Horseradish peroxidase increased activity from 6.3 to 9.9 U/ml by purification steps. It gained 45.77 purification folds at the final step.

Figure 6. Immobilized Peroxidase Extracted from *Nigella sativa* in Calcium Alginate Beads

Table 1. The Purification Steps of Peroxidase from *Nigella sativa*

Sample	Volume (ml)	Enzyme Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification Fold	Yield (%)
Crude Extract	30	1331	0.95	1401	39930	1	100
Concentration by dialysis using Sucrose	10	2444.2	0.7	3491.7	24442	2.5	61.2
Gel Filtration Chromatography using (Sephadex G-150)	18	775	0.2	3875	13950	2.8	35

Immobilization of peroxidase by entrapment method

Crude *Nigella sativa* peroxidase was immobilized by entrapment in calcium alginate (Fig. 6). The immobilization ratio of the calcium alginate entrapped peroxidase reached to 49%. The calcium alginate have several properties for entrapment of *Nigella sativa* peroxidase, it might attributed to the difference between other matrices such as the nature, component and porosity of them. Entrapment of enzyme in alginate was one of the simplest, cheapest and non-toxic that more frequently used method of immobilization, it's also provides mild and physiological condition for enzyme entrapment (21). Concerning to the other studies it was recorded that the immobilization of peroxidase in calcium

alginate was the most favorable method among entrapment methods due to protection the enzyme from change of temperature, osmotic pressure, chemical environment, and chemical reactions, furthermore it was characterized by safety, simplicity, easy, rapid, cheap and offering good mechanical strength (7, 10). The immobilization of peroxidase in calcium alginate is influenced by alginate concentration, enzyme alginate ratio, CaCl_2 concentration and bead size (2). Shaffiqu *et al.*, (26) immobilized peroxidase from *Saccharum spontaneum* leaf on a hydrophobic matrix.

Application of peroxidase

Dyes decolorization: The textile production industry is one of the oldest and most technologically complex of all industries. Textile product has also been increased due to

the increasing demand of the population. Textile mills and their waste water have been increasing proportionally, causing a major problem of pollution in the world. The textile industry accounts for two thirds of the total dyestuff market. During dyeing process approximately 10-15% of the dyes used are released into the waste water. It is recognized as the root cause of environmental pollution (16). The textile dyes, along with a large number of industrial pollutants, are highly toxic and potentially carcinogenic (27), so that they are related to environmental degradation and various diseases in animals and humans (16). The various textile dyes (textile yellow, textile red, textile black and textile blue) degradation capability of peroxidase (crude, purified and immobilized) were studied at dyes concentration of 30 mg/l, in pH 5.0 at 37°C after 24 hr as seen in table (2) and (Fig. 7, 8 and 9). Absorbance of each dye was recorded at suitable wave length for each one. Results showed that the value of each absorbance was decreased through the incubation time increases and stabilized after 3 hr and even after 24 hr, compared with absorbance of the control which didn't changed during 24 hrs, these results indicated that peroxidase have ability to degrade different dyes. Results in the table (2) shows significant differences in rate of degradation which is due to each dye has different structure that effects the degradation capability of peroxidase enzyme. Textile red exhibited higher degradation capacity with purified peroxidase and showed maximum removal extent of 94.6 % after 3 hrs with initial purified peroxidase, followed by textile black dye, textile yellow and textile blue with removal efficiency of 92%, 80.3% and 77.5 % respectively (table 2). Whereas, crude peroxidase has proximately same efficient to degrade dyes compared with purified enzyme. And because of the similar efficiency of the purified with the crude enzyme in removing the dyes, this is why the crude enzyme was immobilized and used to remove the textile dyes. Kokol *et al*, (19) observed that crude peroxidase without purification is cheaper and

more stability comparing to purified peroxidase. Nevertheless, using crude peroxidase would reduce the cost of enzymatic-based decolorization process in industrial scale. These encouraging results suppose a step forward towards the industrial application of peroxidase. Also, these dyes were not decolorized at the same extent that may be due to the difference of the redox potentials and the suitability of their steric structure with the active site of the enzyme (29). The extent of decolorization activity depends on the source of the enzyme and the chemical structure of the dye (1, 9). It has been reported that the chemical structures of dyes largely influence their decolorization rates with peroxidase and that its decolorization efficiency was limited to several azo dye structures (8). The result also exhibited that the red dye recorded the higher degradation rate by immobilized enzyme with maximum removal efficiency of 81% after 3 hrs compared with other 3 dyes. This variation in the ability of the peroxidase for dyes degradation due to the complexity of the dye structure which influence on the degradation rate by peroxidase. Hussein (15) noted that heterocyclic dyes were resistant to enzymatic oxidation. Shaffiqu *et al*, (26) was immobilized the purified peroxidase from *Saccharum. spontaneum* leaf on a hydrophobic matrix. Four textile dyes, Procion Navy Blue HER, Procion Brilliant Blue H-7G, Procion Green HE-4BD, and Supranol Green, at an initial concentration of 50 mg/L were completely degrade Supranol Green and Procion Green HE-4BD (100%) dyes within 1 h, whereas Direct Blue, Procion Brilliant Blue H-7G and Chrysoidine were degraded more than 70% in 1 h. within 8 h by the enzyme immobilized on the modified polyethylene matrix. Peroxidase of *Lpomea* leaf degraded 50 mg/L of the dyes Methyl Orange (26%), Crystal Violet (36%), and Supranol Green (68%) in 2-4 h and Brilliant Green (54%), Direct Blue (15%), and Chrysoidine (44%) at the 25 mg/L level in 1 to 2 h of treatment.

Table 2. Dyes Degradation % by Peroxidase enzyme (Crude, Purified and Immobilized) from *Nigella sativa* at 37°C in pH 5.0 after 24 hr.

Dyes	Dyes Degradation		
	Crude Peroxidase	Purified Peroxidase	Immobilized Peroxidase
Textile Reactive Yellow	76.9	80.3	70
Textile Reactive Red	88.7	94.6	81
Textile Reactive Blue	88	77.5	56.4
Textile Reactive Black	91	92	72

Figure 7. Dyes Decolorization by Crude Peroxidase Extracted from *Nigella sativa* in pH 5.0 at 37°C with a concentration of 30 mg/l after 24 hr **Figure 8. Dyes Decolorization by Purified Peroxidase Extracted from *Nigella sativa* in pH 5.0 at 37°C with a concentration of 30 mg/l after 24 hr** **Figure 9. Dyes Decolorization by Immobilized Peroxidase Extracted from *Nigella sativa* in pH 5.0 at 37°C with a concentration of 30 mg/l after 24 hr****REFERENCES**

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