DEGRADATION EFFICIENCY OF PHENOLIC COMPOUNDS USING IMMOBILIZED PEROXIDASE PURIFIED FROM SOYBEAN

peroxidase purified from soybean

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ABSTRACT

This study was aimed on purification, immobilization and application of peroxidase enzyme from whole plant of soy bean for the decolorization of textile dyes and degradation of some phenolic compounds. The purification of peroxidase was in two steps include: concentration by sucrose and gel filtration by using sephadex G-150. The purification fold was 4.46 and 2.09 with an enzyme yield of 22.34% and 25.55% for peak 1 and peak 2 respectively. The purified peroxidase enzyme was immobilized by two methods include: covalent linkage immobilization by gluteraldehyde activated chitosan and entrapment by sodium alginate and agarose gel, the immobilization ratio reached to 58.07%, 50% and 11.75% respectively. Crude, purified and immobilized peroxidase were studied with textile dyes (red, blue, yellow and black) after 24 hr, the maximum removal efficiency was with immobilized peroxidase reached to 13.25, 35.12, 14.37 and 26.92 % respectively. Immobilized peroxidase on chitosan was able to degrade some phenolic compounds (tannic acid, naphthalene and gallic acid) during 2 hr, and the degradation efficiency was reached to 95.85%, 79.75% and 33.88% respectively.

Keywords: purification, immobilization, textile dyes, entrapment method, chitosan
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كفاءة التفكيك للمركبات الفينولية بأستعمال البيروكسيديز المقيد والمنقى من فول الصويا

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المستخلص

يهدف البحث الى تنقية، تقييد وتطبيقات انزيم البيروكسيديز المستخلص من نبات فول الصويا في أزالة بعض صبغات معامل النسيج وتفكيك بعض المركبات الفينولية. نقي أنزيم البيروكسيديز المستخلص من النبات الكامل بخطوتين تضمنت التركيز بالسكروز ثم الترشيح الهلامي على عمود السيفادكس (G–150) وكان عدد مرات التنقيه 4.46 و 2.09 بحصيله أنزيميه مقدارها 22.34% و 25.55% لكل من القمه الاولى والقمه الثانيه على التوالي . تم تقييد انزيم البيروكسيديز المستخلص من نبات فول الصويا بأستخدام طريقة التقييد التساهمي على مادة الكيتوسان المنشط بالكلوترألدهايد وطريقة التقييد بالحجز في ماده الجنات الصوديوم وماده الاكاروز حيث وصلت نسبة التقييد الى 58.07%، 50% و 11.75% على التوالي. أختبرت كفاءة الانزيم الخام والمنقى والمقيد بطريقه التقييد التساهمي في تحليل وأزالة بعض صبغات معامل النسيج (الحمراء، الزرقاء، الصفراء والسوداء) وبعد 24 ساعه من المعاملة وجد ان الأنزيم المقيد كان له اعلى كفاءه في ازالة الصبغات وتمثلت النتائج 13.25 و 35.12 و 14.37 و 26.92 % على التوالي. كان لأنزيم البيروكسيديزالمقيد بواسطة طريقة التقييد التساهمي القدرة في تحليل بعض المركبات الفينولية مثل حامض التانك, النفثالين وحامض الجاليك خلال ساعتين، وتمثلت النتائج الخاصه بالتفكيك 95.85 %، 79.75% و 33.88% على التوالي.

كلمات مفتاحية: التنقية، التقييد، صبغات النسيج، طريقة الحجز، الكيتوسان.
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INTRODUCTION

The soybean (*Glycine max*), or soya bean, is a species of legume native to east Asia, widely grown for its edible beans. Fat-free soybean meal is a significant and cheap source of protein for animal feeds, many packaged meals and many other uses. Also soy bean is the best and cheap source for peroxidase enzyme extraction (18). Peroxidase (E.C.1.11.1.7) is one of any number of protein-based enzymes act as catalysts to facilitate a variety of biological processes. Specifically, peroxidase activity involves donating electrons to bind other substrate substances (13). Plants are the rich sources of peroxidases and primarily found in roots and sprouts of higher plants. The rich plant sources of peroxidases are horse radish, potato tuber and beet, while it is also present in higher plants such as turnip, sweet potato, tomato, sour lime, soybean, wheat, carrot, pears, bananas, apricot ,date and sap of fig tree(3). Peroxidases act on the removal of hydrogen atoms most usually from the alcohol groups, which are combined with hydrogen peroxide in order to form molecules of water and oxidized phenolic compounds, acting as detoxifying enzymes and as a cell wall crossing linked enzyme during wounding stress (17). Peroxidase of whole plant purified by gel filteration chromatography using sephadex G-150 column then immobilized using entrapment method(sodium alginate, agarose gel) and covalent linkage by chitosan

MATERIALS AND METHODS

Purification of peroxidase enzyme

Peroxidase was purified from soy bean plant using dialysis method for enzyme concentration, followed by gel filtration.

Enzyme concentration by sucrose

The crude enzyme solution was concentrated by sucrose using dialysis tubes with MWCO=10 KDa, then the enzyme activity, protein concentration, and the specific activity were measured.

Gel filtration chromatography

Separation of enzyme through sephadex G-150 column

The concentrated enzyme by sucrose (5 ml) was passed through sephadex G-150 column. 0.2M potassium phosphate buffer solution, pH 6.5 was used for the elution step with flow rate 15 ml / h with 3 ml for each fraction. The protein fractions were monitored in each fraction at wave length 280 nm, then enzyme activity was measured of these fractions. The effective fractions were collected, then volume was measured, activity and protein concentration were estimated, then the volume was collected and distributed in tubes, freeze and kept for subsequent experiments.

Immobilization of peroxidase by entrapment methods in sodium alginate (3%)

Two ml of purified peroxidase (60.06 U/ml) was mixed with 10 ml of sterile sodium alginate solution (3%) and then stirred gently for 10 minutes. The mixture obtained was extruded drop wise through a sterile syringe (10 ml) into 0.2M of cooled $CaCl_2$ solution to obtain small beads with 3 mm diameter and kept for 1 hr. Then the beads were washed with cooled $CaCl_2$ solution to remove the non-immobilized enzyme. Finally the calcium alginate beads of enzyme were stored in 0.2 M $CaCl_2$ at 4 ^{o}C and the Immobilized enzyme activity was determined **(**22).

Immobilization of peroxidase by entrapment method in agarose Gel (1%)

Two ml of purified Peroxidase (60.06 U/ml) was mixed with 10 ml of sterile agarose gel solution, shaking well for few minutes and poured into sterile petri dish and allowed to solidify. After solidification, cubes 3 mm were cut and washed with D.W. to remove non-immobilized enzyme. Then the squares of agarose-bounded enzyme were stored in 0.2 M of sodium phosphate buffer pH 7 at 4^{o} C and the Immobilized enzyme activity was determined

Immobilization of peroxidase by covalent method by chitosan

One gram of chitosan was added to 10 ml of 2% gluteraldehyde solution, stirred gently and mixed for 2 hr, at 4 ^{o} C followed by an overnight incubation. The gluteraldehyde bounded chitosan was washed extensively with water to remove the unbounded gluteraldehyde. Then gluteraldehyde bounded chitosan was mixed with 2 ml of purified enzyme solution (60.06 U/ml) at 4 ^{o} C overnight for enzyme immobilization. Finally the resulted chitosan - gluteraldehyde – peroxidase conjugates were separated and

washed and Immobilized enzyme activity was determined (8).

Enzyme immobilization %

To calculate the activity of immobilized peroxidase must define total peroxidase activity and non-immobilized peroxidase activity (found in the water that were used to wash the peads of sodium alginate, pieces of agarose and chitosan-bounded-gluteraldehyde), the activity of immobilized enzyme was calculated according to the follwing equations: Immobilized Enzyme activity = Total enzyme activity – non immobilized enzyme activity

Enzyme Immobilization (%) = Immobilization enzyme nits / Total enzyme units*100

Application of peroxidase enzyme

Dyes decolorization

For decolorization experiments, four textile dyes (red, blue, yellow and black) obtained from Al-diwaniyah textile factory, were used in the present study. The reaction mixture for the degradation of dyes contain 5 ml (50 µL /l) from each dyes and 1 ml of 4 U/ml of crude, purified enzyme and 8 U/g for immobilized peroxidase separately (2). In the control experiment the distilled water was used instead of enzyme. The degradation of a specific dye was calculated in different time incubation (0, 1, 2, 3 and 24) hr and the percentage of removal efficiency for each dye was calculated by the absorbance at λ max (24).

Decolorization(%)= [(Initial Absorbance – Final Absorbance) / Initial Absorbance] x100

Degradation of phenolic compounds

Phenolic compounds such as Gallic acid, tannic acid and naphthalene were prepared at concentrations 50 mg/l. The reaction mixture for sample consists of 5 ml of each compound and 0.5 g of immobilized enzyme (4 U/ml). A blank contained distilled water instead of enzyme. Both the sample and blank were incubated at 40°C in water bath for 2 hr. sample was analyzed using Gas chromatography (GC) technique. The degradation % was calculated based on the following equation:

Degradation (%) = [(Initial Concentration – Final Concentration) / Initial Concentration] x100

RESULTS AND DISCUSSION

Purification of peroxidase

The crude peroxidase was purified from the unwanted proteins and other components using two steps included the concentration by sucrose, and gel filtration using (Sephadex – G150) as follows:

Enzyme concentration by sucrose

The crude extract was initially subjected for concentration by sucrose, 63.97% of peroxidase enzyme was yielded with purification fold 4.23 as shown in Table 1. Sucrose is widely used for the concentration of proteins, it almost used as an inexpensive method of precipitating and concentrating a protein extract. The method for the concentration of 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 (4).

Gel filtration chromatography

The solution of concentrated enzyme was passed through a gel filtration column Sephadex G-150 with the dimensions (21× 1.5) cm, and equilibrium with 0.2 M of potassium phosphate buffer (pH 6.5).The result indicated presence of two peak of protein in the eluted fractions from the column that compatible with two peak of peroxidase activity in fractions (4-9) and (11-21). In this step, the specific activity reached to 13045.33 and 6103 U/mg protein for the two peaks respectively with a purification fold 4.46 and 2.09 and yield reached to 22.34 % and 25.55 % respectively as shown in Table 1. Elisete da Silva *et al*., Purified peroxidase that isolated from the pulp of ripening papaya fruit, soluble and bound peroxidase activities increased 2.5 and 4.2 fold respectively and the polyacrylamide gel electrophoresis of the purified preparations revealed that both soluble and bound enzymes were highly purified. The soluble and bound forms had a *Mr* of 41.000Da and 54.000Da, respectively (12).Sessa and Aanderson purified soybean peroxidase by gel filtration on Bio-Gel P-60; purification fold was 2.37 with yield 28.54% (19). While Zia *et al.*, purified peroxidase from apple and orange seeds respectively by using sephadex G-75, the specific activity was 9.2U/mg and 18.16U/mg protein with

purification fold 1.54 and the yield was 18.53% respectively (25).

Table 1. Purification steps of peroxidase from soybean

Purification step	Volume (ml)	Activity (U/ml)	Protein conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification Fold	Yield (%)
Crud Enzyme	18	875.75	0.3	2919.16	15763.5	1	100
Concentration by sucrose	5	2016.93	0.163	12373.80	10084.65	4.23	63.97
Gel Filtration (SephadexG-150) Peak 1	18	195.68	0.015	13045.33	3522.24	4.46	22.34
Gel filteration (SephadexG-150) Peak 2	33	122.06	0.02	6103	4027.98	2.09	25.55

Immobilization of peroxidase enzyme

Two types of immobilization methods were tested represented by entrapment in sodium alginate and agarose (Fig 3. and.Fig 4.), and the second method represented by covalent method to gluteraldehyde activated chitosan(Fig 5). Immobilization of peroxidase in gluteraldehyde activated chitosan was more suitable than other methods, the immobilization ratio of the chitosan linkage peroxidase reached to 58.07%. Immobilization of enzymes on chitosan carrier is commonly carried out via multipoint covalent attachment at high ionic strength, because it has been postulated that, in a first step, a salt-induced association between the protein and a very high density of reactive groups on the support surface with takes place(23).since immobilization ratio of the sodium alginate entrapped peroxidase reached to 50%, which was higher than that of agarose (11.75%), therefore the sodium alginate was more suitable than agarose gel. It can be concluded that sodium alginate was more suitable matrix for immobilization of soy bean peroxidase than agarose gel, this might attributed to the difference between these matrices such as the nature, component and porosity of them. Entrapment of enzyme in alginate was one of the cheapest, simplest, non-toxic that more frequently used method of immobilization, it's also provides mild and physiological condition for enzyme entrapment (15) and protection the enzyme from temperature change, osmatic pressure, chemical reactions and chemical environment. Furthermore, it was characterized by safety, simplicity, easy, rapid, cheap and offering good mechanical strength (7, 11). Bindhu found that 62% of horseradish peroxidase was immobilized on chitosan when 1-ethyl-3-(3-dimethylaminopropyl carbodiimide) was used as the peptide coupling agent(5).While Miao and Tan found that 85% of horseradish peroxidase activity after 30 d of storage in a phosphate buffer at 4 °C using chitosan film crosslinked with glutaraldehyde(14). Bilal etal., found that the immobilization ratio of purified (MNP) from *Ganoderma lucidum* IBL-05 in agar-agar support was4.0%(6).

Application of peroxidase

Dyes decolorization

Four textile dyes (textile red, textile blue, textile yellow and textile black) were used to determine the degradation capability of peroxidase(crude, purified and immobilized) at concentration 50 µL/l, after 24 hr. as revealed in Table 2 the Absorbance of each dyes 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 did not change during 24 hr., these results indicated that peroxidase have ability to degrade different dyes. Textile blue exhibited higher degradation capacity with immobilized peroxidase and showed maximum removal extent of 35.12 % after 24 hr., followed by textile black with removal efficiency of 26.92 % for each one (table 2). While the degradation capacity of textile red and textile yellow were reached to 13.25% and 14.37%

respectively for immobilized peroxidase. Whereas, crude peroxidase has proximately same efficient to degrade dyes compared with purified enzyme. These dyes were not decolorized at the same extent this due to the difference of the redox potentials, the suitability of their steric structure with the active site of the enzyme and the complexity of the dye structure which influence on the degradation rate by peroxidase (21).The extent of decolorization activity depends on the source of the enzyme and the chemical structure of the dye (1,10). Ollikka etal., noted that heterocyclic dyes were resistant to enzymatic oxidation (16). Shaffiqu with coworkers found that purified peroxidase from *Saccharum spontaneum* leaf could completely degrade Supranol Green and Procion Green HE-4BD dyes (100%) within 1 h, whereas Direct Blue, Procion Brilliant Blue H-7G and Chrysoidine were degraded > 70% in 1 h. Peroxidase of *Ipomea*leaf degraded 50 mg/L of the dyes Methyl Orange (26%), Crystal Violet (36%), Supranol Green (68%) in 2-4 h and Brilliant Green (54%), Direct Blue (15%), and Chrysoidine (44%) at the concentration 25 mg/L in 1 to 2 h of treatment1. The *Saccharum* peroxidase was immobilized 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 degraded within 8 h by the enzyme immobilized on the modified polyethylene matrix (20).

Table 2. Dyes degradation% by peroxidase enzyme (crude, purified and immobilized) from soybean after 24 hr

Dyes	Dyes Degradation (%)		
	Crude Peroxidase	Purified Peroxidase	Immobilized Peroxidase
Red	8.44.	2.52	13.25
Blue	2.15	3.26	35.12
Yellow	3.2	1.58	14. 37
Black	4.91	7.44	26.92

Degradation of phenolic compounds

Various phenolic compounds Tannic acid, Naphthalene and Gallic acid degradation capability of immobilized peroxidase were studied at concentration of 50 mg/l, after 2 hr. The phenolic compounds degradation was measured by (GC).The results in the table (3) showed that the maximum degradation capacity of tannic acid by immobilized peroxidase was 95.85 % followed by 79.75 % and 33.88 % removal efficiency of naphthalene and gallic acid respectively. Colin Flock studied the removal of phenols and chlorophenols by soy bean peroxidase. Chlorophenol were added to the reactor with the optimum amounts of seed hulls and hydrogen peroxide and allowed to mix for one hour. The 2,4-dichlorophenol had the highest removal with over 40% being removed. The other four constituents were paired off with the catechol and hydroquinone having over 80% remaining while the phenol and chlorophenol had a negligible amount removed (around 5%)(9).

Table 3. Degradation efficiency (%) of phenolic compounds by immobilized peroxidase enzyme extracted from soybean

Phenolic compounds	Degradation Efficiency %
	Immobilized peroxidase
Tannic acid	95.85
Naphthalene	79.75
Gallic acid	33.88

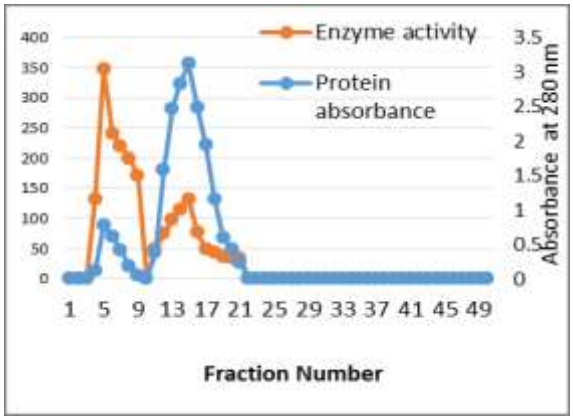

Figure 1. Gel filtration chromatography using sephadexG-150 column with dimension (21*1.5) cm for purification the peroxidase from soybean equilibrate with phosphate buffer pH.6.5, flow rate 15ml/hr. and fraction volume 3ml

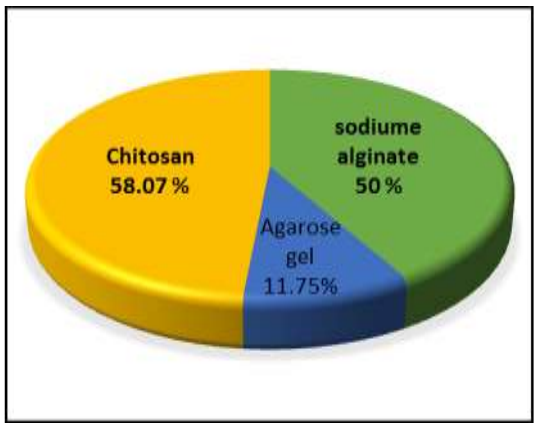

Figure 2. Enzyme immobilization% of purified peroxidase by entrapment method (sodium alginate and agarose gel) and covalent method using chitosan

Figure 3. Immobilized peroxidase in agaros

Figure 4. Immobilized peroxidase in Sodium alginate

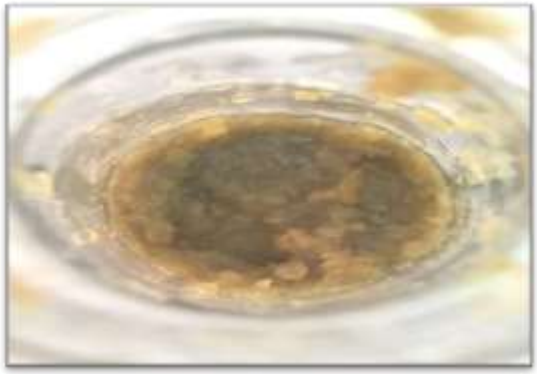

Figure 5. Immobilized peroxidase on gluteraldehyde activated chitosan

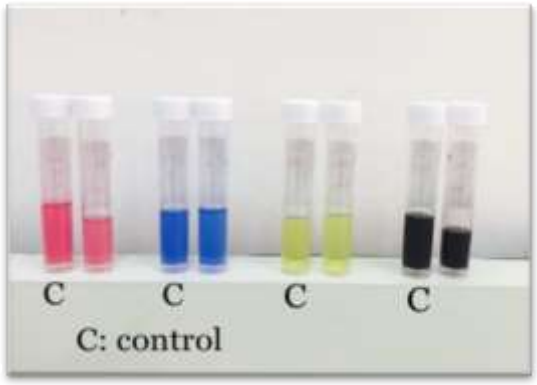

Figure 6. Dyes decolorization by crude peroxidase extracted from soybean, at a concentration of 50 µL /l after 24 hr

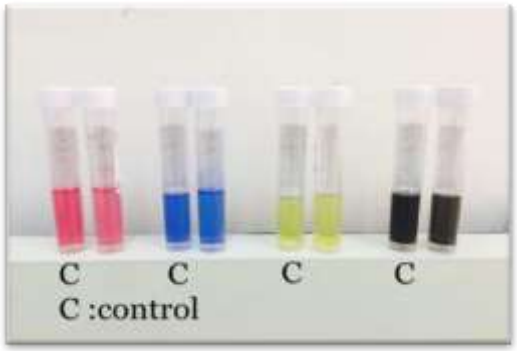

Figure 7. Dyes decolorization by purified peroxidase from Soybean, at a concentration of 50 µL /l after 24 hr

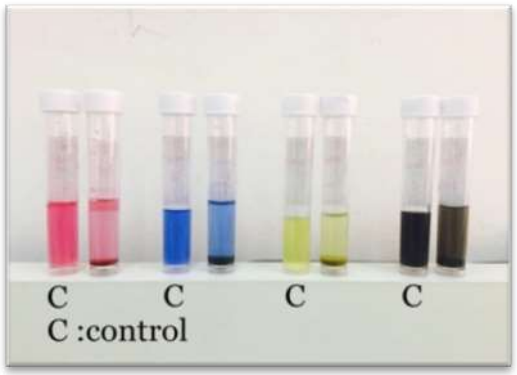

Figure 8. Dyes decolorization by immobilized peroxidase purified from soybean, at a concentration of 50 µL /l after 24 hr

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