Journal of Zankoy Sulaimani (JZS), 2000,Vol.3(1), 1-13

Separation and characterization of polyphenoloxidase (PPO) from Melissia officinalis

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ABSTRUCT

Polyphenol oxidase (ppo) of Melissia officinalis was isolated and purified by gel filtration and ion exchange chromatography. Three isoenzymes were separated.The isoelectric points and some kinetic properties of these isonzymes are reported.

INTRODUCTION

Melissa efficinalis leaves used for decades of generation in the north of Iraq by mountaineers who suffer from infertility. They believed that they make them fertile. Literature survey shows that this plant has a noticable effect in decreasing prolactin (1) It is reported that decrease of prolactin is due to polyphenols, and it is mentioned that polyphenol oxidase has a role in formation of these compounds, since the enzyme inhibition by ascorbic acid, heavy metal ions and temperature treatments has resulted in deactivation of the hole process of reduction of prolactin level (2). The present work aimed of the extraction, separation and purification of the enzyme (ppo) by different methods using calcium acetate, gel filtration on sephadex G-75, chromatography on DEAE sepharose CL-6B. Charactrization of enzyme also has been done to determine the pH- optimum, Temp. optimum, effect of inhibitors, substrate specificity, km and isoelectric focusing. Work is in progress in our laboratory to identify the effective polyphenols produced by the action of purified (ppo) enzyme.

MATERIAL AND METHODS

Melissa officianls were harvested in July from of Eribil city in the north of Iraq the leave were stored at (-20 °C) in freezer. In order to disruption of the membranes and cell walls:-of the tissue of the plant leaves 200g leaves were frozen in liquid nitrogen and ground while still frozen through a Wiley mill.

Enzyme purification

Acetone dried powder leaves of M. officials (20g) were homogenized in a warning blender with 400ml of chilled Acetone. The suspension was immediately filtered through a buchner funnel and the powder washed further with 100ml of cold acetone, air dried and stored at 0^0C.

Extraction of enzyme

10g of the powder was treated with and without phenymethyl sulfonyl chloride (PMSC) in 65ml of 0.1M phosphate buffer, pH 7.2 by constant stirring for 30 min. The extract was filtered through cheesecloth and centrifuged at 4000 g for 10 min. in refrigerated centrifuge.

Calcium acetate treatment

To 60ml of the clear supernatant, 300mg of calcium acetate were added to remove The polyphones that were left in the powder after acetone treatment. After stirring for 15min then centrifugation, the clear supernatant was collected and the Precipitate discard. The supernatant solution representing the partially purified Enzyme (ppo) at 0^0 C against 0.001M phosphate butter (pH7.2)for 6hr to remove Calcium acetate then freeze-dried (partially purified ppo)

Gel filtration on sephadex G-75

The partially purified ppo was chromatographed on scphadex G-75 (pharmacia fine Chemicals) using a 2.5x 54 cm column which was equilibrated with 0.05M Phosphate buffer (PH7.2), fifty mg of ppo was applied to the column. Elution was carried out with 0.05M phosphate buffer (PH 7.2) at a rate of 60ml/hr, 8ml fractions were collected using the 2070 Ultrorac fraction collector and the results recorded with the LKB 2210 Recorder. An aliquot from each fraction and from pooled fraction comprising the activity peaks was assayed for enzyme activity. The column was maintained 4°C. The bed void volume was measured using blue dextran 2000(3).

Chromatography on DEAE sepharose CL-6B:

Fraction Nr.30 to 50 from filtration column were concentrated to a volume (10- 12ml) containing 40mg protein and applied individually to a 2.5 x 25 cm DEAE-sephorse CL- 6B column (pharmacia fine chemicals, Sweden) prepared and equilibrated with the starting buffer (0.05Mphosphat,PH7.2) and washed with the same buffer (300ml).The column was then eluted with a linear gradient of phosphate buffer formed (200ml of 0.05M.PH7.2) in the mixing chamber and 200ml of 0.4M phosphate, pH 7.2 in the reservoir using the pharmacia (GM) gradient mixer (pharmacia Sweden).A flow rate of 12 ml/hr was used and 5ml fractions were collected. The major DEAE-sepharose CL 6B pooled fractions obtained were then separately dialyzed against 2 liters of 0.001 M phosphate buffer, pH 7.2 for 24 hr with two changes and concentrated to a small volume (5-7ml) using the freeze-dryer (purified enzyme).

Enzyme assay:

A modified method of Mayer et. al. (4) was followed for assay of the enzyme. The reaction mixture contained 1ml of 0,1 M phosphate buffer (pH7.20), 0.5ml distilled water and 0.5ml enzyme (protein range 2.25-10mg depending on the fraction used). The mixture was incubated for 5min At 25°C ,then added 1ml of Substrate (catechol 9.9 mM, the cateatechol dissolved in the same buffer). The control contains the solvent mentioned.

The enzyme blank cuvette contained all generals except the substrate. The enzyme activity was carried out with pu 8800 UV.VIS spectrophotometer. The specific activity unit is equal to 0.01 OD at 395 nm per mg protein.

Protein determination:

Protein concentration was determined by the method of lowry el.al(5) with bovine serum albumin as standard.

Determination of proteolytic activity:

To detect the presence of proteolytic activity in the leaf extract the method of Whitaker (1957) was used (6). The proteolytic inhibitor phenyl methyl suphonyl choride was dispersed into the extract to a final concentration of 0.5m M -1mM to inhibit the proteolytic enzymes that occur widely in these tissues. The specific activity is equal to 0.01 OD at 280nm/min/mg protein.

Effect of inhibitors:

To examine the effect of inhibitors on ppo, reaction mixtures containing constant amounts of the enzyme and substrate (9.9mM) were rum in the presence of four different inhibitors.
The reaction was followed and the rate of the substrate was determined according to the method of Maye, et.. al (4). The inhibitors were incubated at 25°C for 30 min with the enzyme preparation before the addition of the substrate.

Isoelectric focusing:

DE.I,DE.II and DE.III peaks which were obtained from chromatography on DEAE-Sepharose CL-6B were concentrated by Amicon high performance-channel filtration system TCFIO and dialyzed against 1% Ampholine solution; thereafter each peak concentrate was isoelectrically focused on a thinlayer polyacrylamide gel using the LBK 2117 Multiphor and the LBK 2103 power supply (LBK-producer AB,Bromma,sweden).Ready polyacrylamide gels, LBK 1809 Ampholine PAGplate, pH range 3.5-9.5 were used. lsoetectrie focusig was carried out as described by LBK-pharmacia application No. 269 (1977).

RESULTS AND DISCUSSION

Figure (1) shows the elution pattern of the enzyme ppo from Sephadex G-75 column. The second peak in the elution pattern strongly retarted.

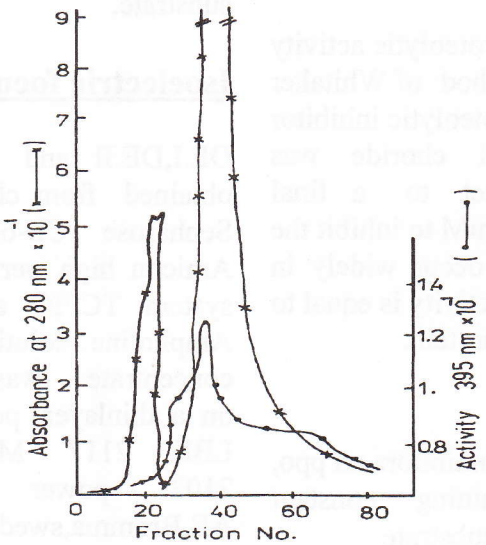

Fig.(1) sephadex G-75 chromatography of partially purified ppo. The enzyme was eluted with 0.05M phosphate buffer, pH 7.2,
x.....x obosrbance at 280nm,. ---- . ppo activity (units/ml).

The delay may be due to heterocyclic or homocyclic aromatic amino acid residues in the enzyme molecule (3). About 75% of the total enzyme activity was recovered. The purification factor at this stage was 2.8 (table1).

Table (1) Purification chart of M. officinal PPO

Purification Steps	Vol. ml	Activity unit/ml	mg/ml	Specific Activity Unit/mg	Total activity	Recovery %	Fold of purification
1-Extraction (from acetone powder)	40	140	64.8	2.16	5600	100	1
2-Calcium Acetate treatment	34	128	36.0	3.55	4352	77.4	1.64
3-Gel filtration on sephadex G-75	54	77.4	12.6	5.99	4180	74.6	2.8
4- lonexchange chromatography on DEDE-Sepharose Cl-6B							
Peak 1	75	25	0.28	89.3	1875	33.44	41.3
Peak 2	43	21	0.25	84	903	16.14	39.0
Peak 3	35	24	0.29	82.7	840	15.4	38.0
Peak 4	18	30	0.4	75	540	9.63	34.0

Fractions Nr.30-50 from the sephadex G-75 column were applied to DEAE-Sephades CL-6B, peaks with enzyme activity recognized (fig2). The specific activity of the four peaks ranged 75-89.3 and the purification folds were 41.3-34 for peaks 1-4 (table 1). peak 1 was eluted with about 0.17M buffer and peak 2 with 0.25 M buffer.

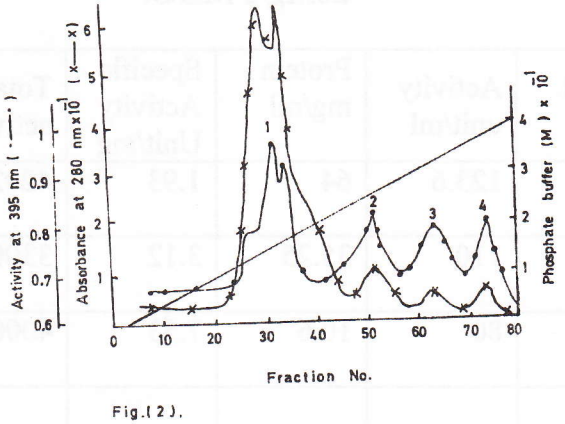

Fig.(2).

Fig. (2) Chromatography of (fraction Nr.30-50 obtained from gel filtration on DEAE-Sepharose CL-6B. The enzyme was eluted with linear phosphate gradient (0.05-0.4M) pH 7.2 x ----x absorbence at 280 nm, . ---- . ppo activity (units /ml) PMSC was not added to the original leave extract.

Effect of pmsc on prteolytic enzymes

Table (2) shows the activity of proteolytic enzyme in crude extract before using of PMSC and upon using PMSC as inhibitor of these enzymes, it shows that there was no proteolytic activity upon using lmM of PMSC.

Table (2): shows the activity of ppo crude extract before and after using PMSC as inhibitor.

PMSC Conc. (mM)	Activity unit/ml	Protein Conc. mg/ml	Specific activity unit/mg protein
Zero	78.8	13.12	6.00
0.5	7	13.13	0.53
1	0.0	13.1	0.00

Table(2) and fig (2) indicate that the extract of M. officinalis leaves contained protease activity. Four (PPO) forms, in addition to a clear shoulder on peak 1 (Fige2) were isolated from the extract by ion-exchange chromatography on DEAE-Sepharose Cl-6B however when the protease inhibitor, phenylmenthylsulfonyl chloride (PMSC) was used , no protease activity observed and only three ppo forms were isolated by ion-exchange chromatography as in fig.3, table (3).

Table (3): The activity and purification steps of PPO of the extract upon using PMSC.

Steps	Vol. ml	Activity unit/ml	Protein mg/ml	Specific Activity Unit/mg	Total activity	Recovery %	Fold of purificatin
1-Extraction (from acetone powder)	34	123.6	64	1.93	4202.4	100	1
2-Calcium Acetate treatment	30	110	35.25	3.12	3300	78.5	1.62
3-Gel filtration on sephadex G-75	50	80	10.6	7.53	4000	95.2	4
4- lonexchange chr. on DEAE-Sepharose CL-6B							
DEI	15	20	0.36	83.3	450	107	43.2
DEII	15	25	0.27	92.6	375	8.9	47.98
DEIII	80	26	0.4	65	2080	49.5	33.68

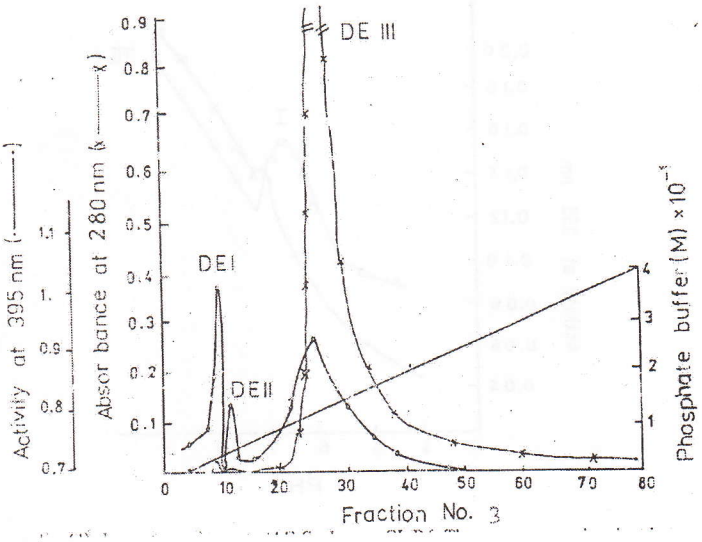

Fig (3) chromatography on DEAE-Sepharose CL-B6. The enzyme was eluted with linear phosphate gradient (0.05-0.4M) PH 7.2 x ----- x absorbance at 280 nm, . ----. ppo activity (unit/ml), PMSC was added to leave extract.

pH PROFILE

Many PH optima for polyphenol oxidase are reported in the linerature depending upon the tissue substrate.

The pH activity profile of the purified ppo (using the peaks DE.I,DE.II and DE.III from DEAE-Sepharose CL-6B) was determined between PH 3.5 and 9 as shown in fig (4). The pH optimum was 6.8, 6.6 and 7 for peak l,ll and lll respectively. Generally the enzyme is active between pH 5 and 8. In alkaline pH, above pH8, one should be careful of endogenous nonenzymic oxidation (8), this is the reason that the activity is raised above pH 7.

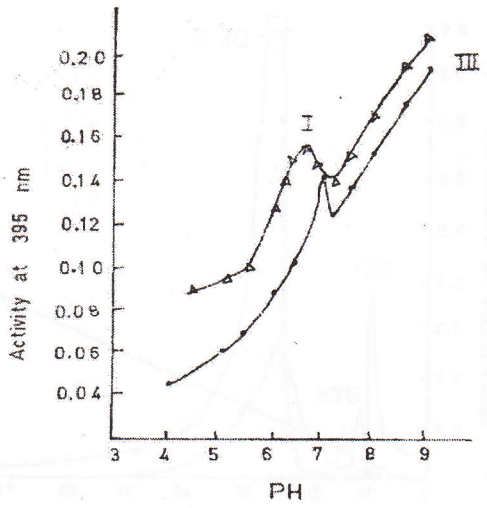

Fig (4) pH dependency of polyphenol oxidase activities at 25°C. The buffer were: citrate phosphate pH 3.5-6; phosphate Na-Na_2 pH 6-7.5; glycine-NaOH pH 8-9.5 (activities were determined at 395nm after reaction for 5min with catechol (9.9mM) as substrate).

The effects of divalent cations on ppo activity

The effect of various cation on the activity of the Melissa officinal polyphenol oxidase is shown in table (4). Addition of Cu^{+2},Co^{+2}or Zn^{+2} ions resulted in an increase in the initial rate of the enzyme reaction while addition of Mn^{+2}, Mg^{+2} or Ni^{+2} had little effect on the initial velocity.

The effect of cupric ions may indicate the conversion of the apoenzyme into holoenzyme (7). The role of cobalt ions on the enzyme reaction remains obscure.

Table (4) :Effect of divalent cations on PPO activity. *

Divalent cations	Absorbance at 395nm	%
—	0.065	100
Co^{+2}	0.109	167.7
Cu^{+2}	0.08	121.5
Zn^{+2}	0.075	115.4
Mn^{+2}	0.6	98.77
Mg^{+2}	0.059	90.77
Ni^{+2}	0.056	89.2

*The absorbance determined was determined at 395 nm after reaction for 5min. at 25°C and pH 7 with catechol (9.9mM) as substrate. Peak DE II (with highest activity) from ion exchange chromatography was used to determine the effect of divalent cations.

TEMPERATURE OPTIMUM

Fig. (5) shows the optimum temperature for the three fraction DE.I, DE.II,and DE.III, they were 30,34 and 40°C respectively.

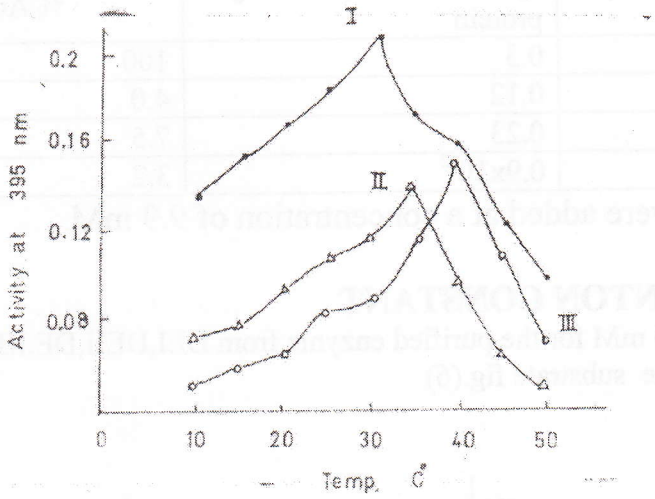

Fig (5) ; The activity determined at 395 nm after enzyme reaction at pH7 and various temperature for 5min with catechol (9,9mM) as substrate.

EFFECT OF INHIBITOR'S

Influences of some inhibitors on activity of the Melissia officinalis polyphenol oxidase in crude enzyme extract are summarized in table (5) concerning ascorbic acid, Sulphohydryl compounds, Sodium chloride and azide are in line with reported work (9,10,11).

Table (5): The effect of various inhibitors on polyphenol oxidase present in the crude extract.

Inhibitors	Conc. required for 50% inhibition(mM)
Thiourea	1.7
Sodium Azide	5.2
Ascorbic Acid	0.5
Sodium Chloride	$1x10^{-3}$

SUBSTRATE SPECIFICITY

The substrate specificity of purified enzyme (peak ll from DEAE-Sepharose CL-6B) is shown in table (6). Maximum activity was demonstrated with chlorogenic acid and Catechol among the various substrate studied

Table (6); Substrate specifity of polyphenol oxidase.*

Substrate	Specific activity unit/mg protein	% Activity
Chlorogenic acid	0.3	100
Catechol	0.12	4.0
P-cresol	0.23	7.5
Tyrosine	$0.9x10^{-2}$	3.2

* All the substrates were added at a concentration of 9.9 mM.

MICHAELIS – MENTON CONSTANT

The Km was 1, 1.25,1.66 mM for the purified enzyme from DEI,DEII,DE,III, respectively Catechol was used as the substrate fig.(6)

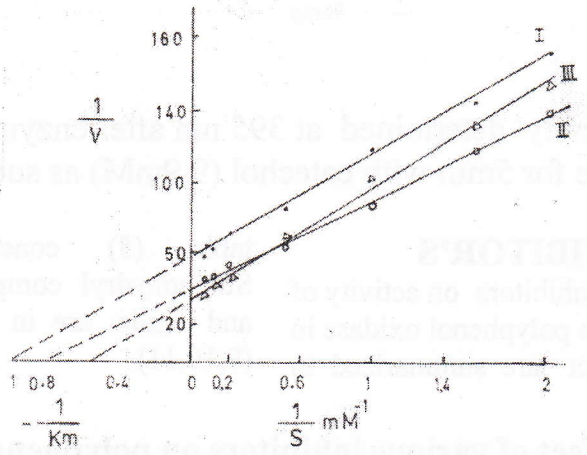

Fig.(6) Lineweaver – Burke plot for ppo obtained from DE. l, DE. ll, DE. lll with catechol as variable substrate.

These Km values were calculated by extrapolation of the approximatively linear part of the lineweaver burk plot Fig.6 for catchol , they were higher when compared with the mushroom ppo enzyme (12) and Tea enzyme (13).

The PI values of three ppo enzyme peaks DE.I,DE.II And DE.III were 4.8,5.2 and 5.4 respectively Fig (7). Isoelectric focusing has been applied to prove that the fractions obtained from DEAE-Sepharose –CL B6 ,

were pure and each fraction contains only one isoenzyme, this indicates that the ppo enzyme from M. officinalis leave extract composed of three isoenzymes only.

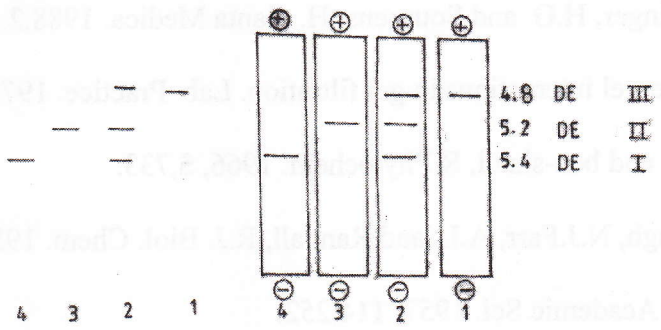

Fig. (7) Focusing on thin-layer polyacrylamide gel
1-Focusing of DE. 111
2-Focusing of all three peaks together
3-Focusing of DE. ll
4-Focusing of DE. l

Acknowledgement:
The auther would like to thank Prof. Kemper at the Institute of pharmacology and toxicology Munster Germany for his faithful discussions.

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پەسەندکرا لە ٢٠٠٠/٥/٢٨ دا

جیاکردنەوەی ئەنزیمی پۆلی (فینۆل اوکسیدیز) وەدەستنیشانکردنی تایبەتیەکانی لە ڕووەکی تورنجاندا. (Melissia officinalis)

د.دلاوەر محمد صابر
کۆلیجی زانست / بەشی کیمیا
زانکۆی سەلاحەدین / هەولێر

کورتە

گەڵای ڕووەکی تورنجان بۆ سەردەرمانێکی دوورودرێژ لە لایەنی دانیشتوانی ناوچە چیاییەکانی کوردستانی عێراقەوە بەکارهێنراوە بەو بڕوایەی کە دەبێتە هۆی نەهێشتنی نەزۆکی . تۆێژینەوە لەبواڕە ئەدەبیەکاندا ئاماژە بۆئەوەدەکات کە کاریگەری لەسەر کەمکردنەوەی هۆرمۆنی پڕۆلاکتین هەیە چونکە پێکهاتەی فینۆلات (polyphenols) ی تیدایە، دەرکەوتوە کە ئەنزیمی پۆلی فینۆل ئۆکسیدیز دەوری لە دروست بوونی ئەوپێکهاتانەدا هەیە . هەروەها دەرکەوتووە کە پەک کەوتنی ئەم ئەنزیمە بەهۆی ترشەلۆکی ئەسکۆربیک وە ئایۆناتی کانزا قورسەکان وە پلەی گەرمی یەوە بووە هۆی ئەوەی کە سەرکەوێتە سەر چالاکیەکانی ئەم ئەنزیمە وەکارکردنی لەسەر نزمکردنەوەی هۆرمۆنی پڕۆلاکتین لە ڕووەکەدا . ئەم تۆێژینەوەیە پتر لەسەر جیاکردنەوەی ئەنزیم وپاڵاوتن ودەستنیشان کردنی تایبەتمەندیەکانی چڕبۆتەوە کارکردنیش لەسەر دەستنیشان کردنی پێکهاتەی جیاجیای فینۆلەکان بە هۆکاری ئەنزیمەوە دەست نیشان ئەکرێ .

فصل انزيم البولى فينول اوكسيديز وتعيين صفاته فالتقاوي نبات الترنجان Melissia officinalis

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الخلاصة

استخدمت اوراق نبات الترنجان لعقود متعددة من قبل قاطني الجبال في كوردستان العراق ظناً منهم انه يزيل العقم . و تشير الابحاث في الادبيات ان لهذا النبات تأثير خافض لهرمون البرولاكتين نتيجة لاحتواءه على مركبات متعددة الفينولات (polyphenols).

و قد وجد ان لانزيم البولي فينول اوكسيديز دور في تكوين تلك المركبات، حيث لوحظ ان تثبيط هذا الانزيم بواسطة حامض الاسكوربيك و ايونات المعادن الثقيلة و درجات الحرارة قد ادى بالفعل الى القضاء على فعالية الانزيم و فقدان النبات لتأثيره الخافض لهورمون البرولاكتين .

و لقد ركزت هذه الدراسة على فصل الانزيم و تنقيته و تعيين صفاته ، و العمل مستمر لتشخيص مركبات متعددة الفينول التي تنتج بفعل الانزيم.