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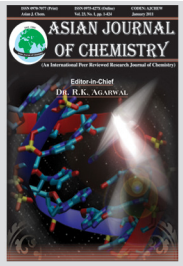

Purification of Paraoxonase (PON1) from Olive (*Olea europaea* L.) and Effect of Some Chemicals on Paraoxonase Activity *in vitro*

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Paraoxonase was purified from olive (*Olea europaea* L.) using sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography. This enzyme was purified 173.4-fold. SDS-polyacrylamide electrophoresis of the enzyme indicates a single protein staining band with an apparent Mr of 45 kDa. The kinetic properties of the purified enzyme were determined. The enzyme exhibited high activity at broad pH (pH 5.0-9.0) and temperature (40 and 70 °C). The purified enzyme remained stable at 4 °C for more than 1 year. Paraoxonase was mostly stable at 40 ºC. Its' activity decreased in 55 % for 1 h at 60 ºC and 20 % for 4 h at 50 ºC. Using paraoxon as a substrate, the K_m and V_{max} values for the purified enzyme were estimated to be 3.76 mM and 131.5 µmol L dak^{-1}, respectively. The activities were strongly inhibited by Hg^{2+} and Fe^{3+}, while Cu^{2+}, β-mercaptoethanol, dithioerythritol and SDS slightly activated the enzyme. As judged by catalytic efficiencies, paraoxon is the preferred substrate.

Key Words: Paraoxonase, Purification, Olive (*Olea europaea* L.), Metal ions.

INTRODUCTION

The existence of organophosphatase in mammalian plasma was first reported nearly 50 years ago[1]. Subsequent studies have shown this enzyme to be paraoxonase/arylesterase; PON; aryldialkylphosphatase, EC 3.1.8.1), an organophospahatase with broad substrate specificity, including aromatic carboxylic acid esters such as phenyl acetate[2].

Initial investigation of PON1 focused on its ability to hydrolyze organophosphorus (OP) compounds, playing a major role in the detoxification of these compounds and other artificial substrates, so that it may alter significantly an individual's susceptibility to the toxicity of these chemicals[3].

PON is hydrolyzed that oxidized phospholipids in LDL and HDL particles as well as against homocysteinylation[4-6]. The mechanism by which PON1 exerts its action seems to differ in these cases, as the active center for the hydrolysis of oxidized lipids[7].

A few years ago, it was established that PON1 is a member of a multigene family in mammals that includes at least three members: PON1, PON2 and PON3. The three PON genes show a high similarity at the amino acid level between the mammalian species[8,9]. PON1 and PON3 are expressed primarily in the liver. In contrast, PON2 is widely expressed in a number of tissues, including brain, liver, kidney and testis and it may have multiple mRNA forms[9]. PON1 and PON3 reside in the cholesterol-carrying particles HDL, whereas PON2 is found in many tissues[10].

Paraoxonase enzyme, human, cow, rabbit, rat liver, kidney, small intestine, especially in many tissues and in serum and purified[11,12]. But so far nothing has been talking about the presence of plant enzymes PON. Directly linked with lipid metabolism in human and animal fat, in seeds of the enzyme may also be considered and whether PON is the enzyme were investigated. In preliminary experiments there seemed to mature olive plant olive fruits of PON enzyme affinity chromatography (sepharose-4B-L-tyrosine-1-naphthylamine) and is intended to be purified and characterized. It is also obtained as pure light and the structure of the PON enzyme function in plants, some chemicals in order to investigate the *in vitro* effects on purified enzyme was studied.

EXPERIMENTAL

Paraoxon (O,O-diethyl-*p*-nitrophenylphosphate) was purchased from Sigma Chemical Co. Other reagents were of analytical grade and supplied by Sigma (St. Louis, MO) and Merck (Darmstadt, Germany).

Preparation of affinity gel: After 20 mL of sepharose 4B and 20 mL of water were combined. 4 g CNBr was added to this suspension. The mixture was titrated to pH 11 in an ice bath by stirring with a magnet and maintained at that pH for 8-10 min. The reaction was stopped by filtering the gel on a Buchner funnel and washing with cold 0.1 M $NaHCO_3$ buffer (pH: 10). L-tyrosine by using a saturated L-tyrosine solution in the same buffer was coupled to sepharose-L-tyrosine activated with CNBr. The reaction was completed by stirring with a magnet for 1.5 h. In order to remove excess L-tyrosine from the sepharose 4B-L-tyrosine gel, the mixture was washed with large amount of water. The affinity gel obtained by diazotization of 1-naphthylamine and this was coupled to the sepharose 4B-L-tyrosine. For this purpose, 1-naphthylamine (25 mg) was suspended in 10 mL of ice cold 1 M HCl and to the suspension was added 75 mg of sodium nitrate in 5 mL ice-cold water. After 10 min of reaction, the diazotized 1-naphthylamine was poured into 40 mL of the sepharose-L-tyrosine suspension. The pH was adjusted to 9.5 with 1 M NaOH and, after gentle stirring for 3 h at room temperature, the coupled dark red Sepharose derivative[13] was washed with 1 L of water and then 200 mL of 0.05 M *Tris*-sulfate, pH 7.5.

Purification of paraoxonase from olive (*Olea europaea* L.): All purification procedures were carried out at room temperature unless otherwise stated.

Preparation of olive (*Olea europaea* L.) homogenate: Mature olive (*Olea europaea* L.) seed from Tarsus in Mersin province in the full maturity stage were collected. The olive (*Olea europaea* L.) was stored at -20 ºC. Before use, the olive was thawed and assayed for total protein and PON1 and ARE activities. Thawed olive was gently mixed with 1 M $CaCl_2$ and 10 M NaCl to give a final concentration of 10 mM $CaCl_2$ and 3 M NaCl and left for 0.5 h at room temperature. Any clot was removed by centrifugation (5000 x g, 20 min) at 4 ºC.

Sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography: The homogenate was applied to the affinity column having a structure of sepharose 4B-L-tyrosine-1-naphthylamine and equilibrated with 25 mM *Tris*/HCl (pH: 8.0)/ 10 mM $CaCl_2$. The affinity gel was washed with the solution 25 mM *Tris*/HCl (pH: 8.0)/10 mM $CaCl_2$/3 M NaCl. Olive paraoxonase was eluted with the solution 25 mM *Tris*/HCl (pH: 8.0)/10 mM $CaCl_2$ at 0.5 mL/min. Fractions (3 mL each) were collected and those with the highest PON1 activity were pooled[14].

Enzyme paraoxonase and arylesterase activities assays: Paraoxonase activity was determined at 25 °C with paraoxon (diethyl *p*-nitrophenyl phosphate) (1 mM) in 50 mM *Tris*/HCl (pH 8.0) containing 1 mM $CaCl_2$. The enzyme assay was based on the estimation of *p*-nitrophenol at 412 nm. The molar extinction coefficient of *p*-nitrophenol (ε = 18,290 M^{-1} cm^{-1} at pH 10.5) was used to calculate enzyme activity[15]. One enzyme unit was defined as the amount of enzyme that catalyzes thehydrolysis of 1 µmol of substrate at 25 °C. Assays were performed using a spectrophotometer (Spekol 1300 UV-Vis)[16-18].

The enzyme activity was also measured an arylesterase assay[14]. Arylesterase activity of PON1 was determined at 25 °C with phenylacetate (1 mM) as the substrate in 50 mM *Tris*/ HCl buffer pH 8.0, containing 1 mM $CaCl_2$. The rate of hydrolysis was measured spectrophotometrically at 270 nm. The molar extinction coefficient of phenol (ε = 1310 M^{-1} cm^{-1}) was used for calculation of activity. One unit of arylesterase activity is equal to 1 µmol of phenylacetate hydrolyzed/min.

SDS polyacrylamide gel electrophoresis (SDS-PAGE): Electrophoresis under denaturing conditions was performed at different polyacrylamide gel concentrations of 10 % according to the discontinuous buffer system of Laemmli[19]. Electrophoresis was carried out in vertical slab gels and the runs were performed at a constant current intensity of 15 mA/ plate in the stacking gels and 30 mA/plate in the running gels. The gel was stabilized in the solution containing 50 % propanol + 10 % TCA + 40 % distilled water for 0.5 h. The staining was performed for about 2 h in a solution of 0.1 % coomassie brilliant blue R-250 + 50 % methanol + 10 % acetic acid. Finally, the washing was carried out in a solution of 50 % methanol + 10 % acetic acid + 40 % distilled water until the protein bands were cleared[19].

Molecular weight determination with gel filtration: Sephacryl S-100 was filled into a column (3 × 70 cm). The column was balanced for 4 h with the buffer (0.05 M Na_3PO_4, 1 mM ditihioerythritol, pH: 7) until the absorbance at 280 nm was no longer obtained. The standard protein solutions bovine serum albumin (66 kDa, pepsin 34.7 kDa, trypsinogen 24 kDa, β-lactoglobulin and lysozyme 14.3 kDa) were added to the column. The purified protease enzyme was added into the column and then eluted under the same conditions. The flow rate through the column was 20 mL/h. The results were compared with standard proteins[20].

Determination of protein amount: Protein content of the samples was quantified according to the method of Bradford[21] using bovine serum albumin as standard.

***In vitro* studies for some chemicals:** Working solutions of the following compounds were prepared from stock solutions in 0.1 M *Tris*-HCl pH 8.1 free of calcium and added to samples to obtain the desired final concentration in the range showed for each compound, as indicated in brackets for PON1 respectively: $CaCl_2$, $MgCl_2$, $Hg(NO_3)_2$, $MnCl_2$, $ZnSO_4$, $Cu(NO_3)_2$, $FeCl_3$, dithioerythritol, β-mercaptoethanol, SDS.

Removal of calcium ions by a chelex 100 column: 1 g of chelex 100 (200 mesh) was washed once with double-distilled water and packed into a 3.0 mL polystyrene column. The packed column was equilibrated with 50 mmol/L *Tris*/ HCl buffer, pH 8.0. Subsequently, 1.0 mL of purified PON Q or PON R was passed through the column at a rate of 0.3 mL/ min. Sequential fractions were collected and assayed for arylesterase activity[22].

Inhibition with EDTA: The purified PON enzyme containing 1.0 mmol/L Ca^{2+} in *Tris*/HCl buffer, pH 8.0, was diluted with equal volume of 1 mmol/L EDTA. Arylesterase activities were essentially zero after 18 h of incubation at room temperature[22].

Heat inactivation: PON was incubated in PBS at 60°C for 15 minutes. PON was not precipitated by this treatment.

Blockage of free sulfhydryl groups: *p*-Hydroxymercuribenzoate (PHMB) or iodoacetate (1 to 10 mmol/L) was incubated with PON enzyme for 1 h at 37 ºC in PBS. Excess sulfhydryl agent was removed before incubation, by dialysis, using a Centricon 100 microconcentrator (Amicon)[22].

Statistical methods: The SAS™ for Windows™ 6.11 computer program was used to perform statistical analyses. Data were presented by descriptive analysis (case number, mean, standard deviation). The comparisons between groups were performed by Student's t-test and ANOVA. The $p < 0.05$ probability was accepted as the significance level.

RESULTS AND DISCUSSION

Olive is one of nutritious foods. Olives and olive oil, is an important requirement for the body but can not be synthesized by the body with essential fatty acids that can dissolve only in oil of some essential vitamins (A, D, E, F) is the source. Major risk factor for cardiovascular disease, which causes arteries to the "LDL" make an impact while reducing component, useful in the location and protective "HDL" component does not change the amount[23].

This data was based, also known as the antioxidant enzyme paraoxonase, oily seeds could also be considered and preliminary trials of PON enzyme was firstly identified in olive oily seeds of plants. This study was to determine the presence of plants and PON enzyme purification of the enzyme in the study of plants is important to be first.

Paraoxonase (PON1) enzyme from olive (*Olea europaea* L.) was separately purified by sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography. Specific activities were calculated in both extract and purified enzyme solution for paraoxonase. Paraoxon and phenyl acetate were used as substrates in the determination of activity and the activity-absorbance graph was drawn (Fig. 1). Furthermore, it was determined how much fold this enzyme was purified and the recovery of enzyme is shown in Table-1. The specific activity of the enzyme increased with every step of purification with a minimum loss in quantity, giving a final recovery of 173.4 (Table-1).

SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme. It was carried out in 10 % and 3 % acrylamide concentrations for the running and the stacking gel, respectively, containing 0.1% SDS[19]. A 20 µg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1 % coomassie brilliant blue R-250 in 50 % methanol and 10 % acetic acid, then destained with several changes of the same solvent without the dye. The electrophoretic pattern was photographed (Fig. 2). Then, the molecular weight of the enzyme was determined as 43 kDa by using the gel filtration chromatograph. These results show that the enzyme has one subunit at 45 kDa.

V_{max} and K_M values were calculated by using Lineweaver-Burk graphs. K_M and Vmax values of purified PON1 are 3.76 mM and 131.5 µmol/L min, respectively.

The properties of purified enzyme from olive (*Olea europaea* L.) were determined. For this purpose, optimum pH,

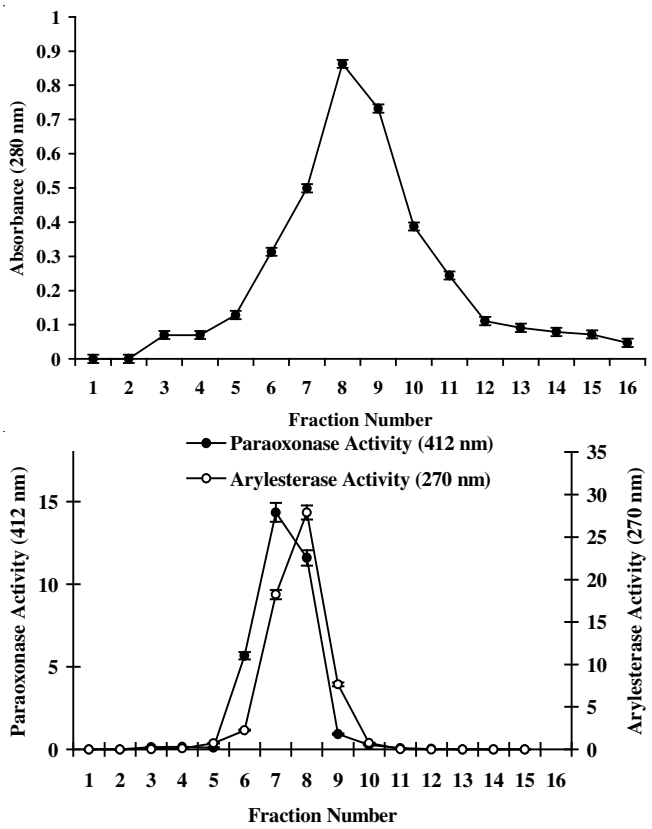

Fig. 1. Graphs of absorbance, paraoxonase and arylesterase activities of paraoxonase enzyme from olive (*Olea europaea* L.)

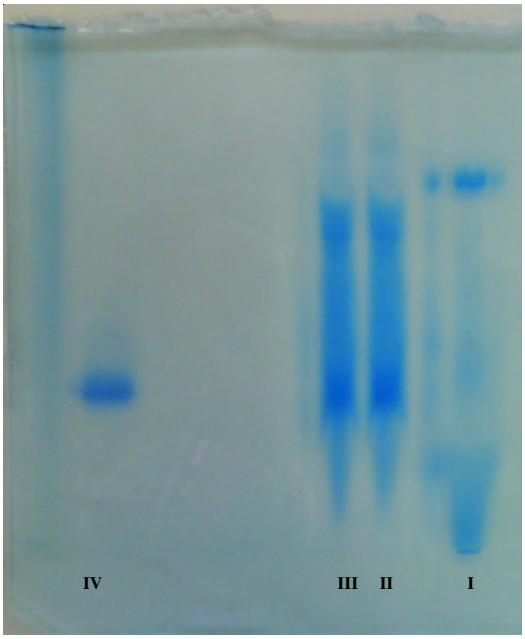

Fig. 2. SDS-PAGE bands of paraoxonase (Lane I: Standard proteins), lane II and III homogenate and lane IV purified paraoxonase from olive (*Olea europaea* L.)

TABLE-1
PURIFICATION OF PARAOXONASE FROM OLIVE (*Olea europaea* L.)

Enzyme fraction	Volume (mL)	Activity (EU/mL)	Total activity EU	Total activity %	Protein (mg/mL)	Specific activity (EU/mg)	Purification (fold)
Homogenate	50	124.5	6225	100	38.2	3.26	-
Sepharose-4B-L-tyrosine-1-naphtylamine	15	158.3	2374.5	38.1	0,28	565.4	173.4

optimum temperature and stability of the enzyme was investigated. In addition, the effects of some substances on enzyme activity were found out. Enzyme inhibition and stability are considered to be the major constraints in the rapid development of biotechnological processes. Stability studies also provide valuable information about structure and function of enzymes.

The stability of PON1 is affected by both physical parameters (pH and temperature) and chemical parameters (inhibitors or activators). The enzymatic hydrolysis of paraoxon also depends on several physicochemical factors. It depends on contact time, enzyme concentration, temperature of incubation and pH.

Similarly, optimal pH studies were investigated for pectin lyase with 1 pH unit increments between 4 and 11 (Fig. 3). The optimal reaction pH for PON1 was 8 and it was active between pH 5 and 10.

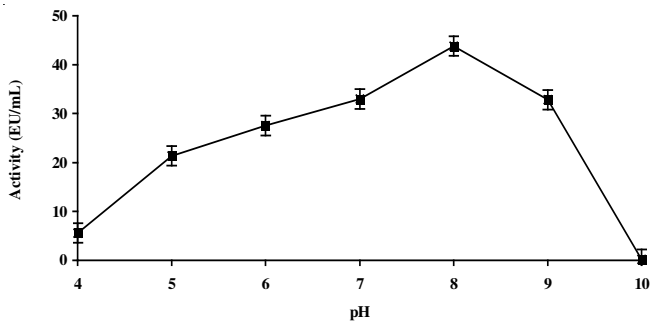

Fig. 3. Effect of pH on the activity of purified paraoxonase from olive (*Olea europaea* L.)

The effect of temperature was investigated between 0 and 90 ºC with 10 ºC increments (Fig. 4) and the optimum temperature was found to be 50 ºC. Enzyme has activity between 20 and 80 ºC. The thermostability study of PON was carried out at temperatures ranging from 40 to 80 ºC (Fig. 5).

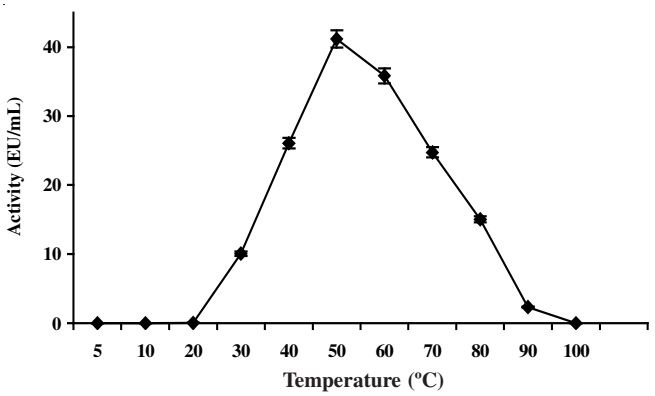

Fig. 4. Effect of temperature on the activity of purified paraoxonase from olive (*Olea europaea* L.)

The purified enzyme appeared to be stable and retained its full activity after 1 h incubation from 40 to 50 °C, but the activity was reduced to 20 % after 1 h at 60 °C. Pectin lyase activity decreased dramatically when the temperature increased above 70 °C with only 13 and 8 % activity remaining 70 and 80 °C, respectively. Thermostability of PON was 40 °C, because it remained nearly full active at 40 °C for at least 24 h.

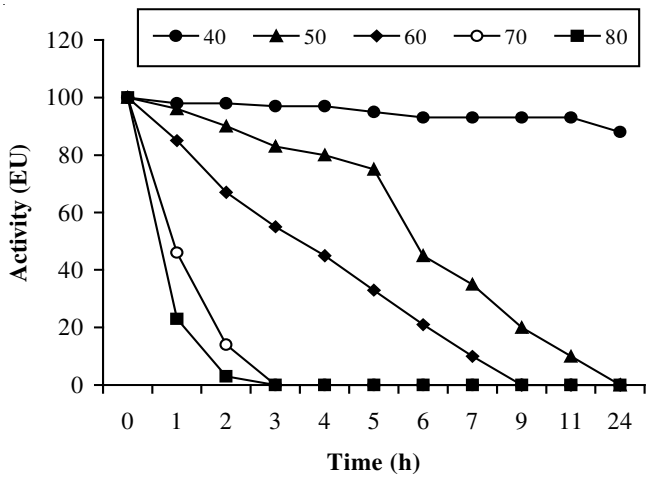

Fig. 5. Temperature stability of the purified PON1 from olive (*Olea europaea* L.)

PON activity was assayed in the presence of different reagents (Table-2). Among the salts tested, considerable loss of activity was observed only with Hg^{2+}, Fe^{3+}, whereas Ca^{2+} and Cu^{2+}, acting somewhat as an enhancer. Inhibition of enzyme activity in presence of Hg^{2+} and Fe^{3+} might be indicative of essential *vicinal* sulfhydryl groups of the enzyme for productive catalysis. The enzyme lost 2.8 % of its activity at 1.0 mM Mg^{2+}, 9.3 % of its activity at 1 mM Mn^{2+} and only 87.7 % of activity was retained at 1 mM Zn^{2+}.

TABLE-2
EFFECT OF ALL KINDS OF COMPOUNDS ON THE ACTIVITY OF PURIFIED PARAOXONASE FROM OLIVE (*Olea europaea* L.)

Chemical compounds	Concentration (mM)	Relative activity (%)
Control	–	100
$CaCl_2$	1	145.2 ± 2.8
$MgCl_2$	1	97.2 ± 3.1
$Hg(NO_3)_2$	1	2 ± 0.1
$MnCl_2$	1	90.7 ± 4.4
$ZnSO_4$	1	87.7 ± 0.4
$Cu(NO_3)_2$	1	121.2 ± 5.1
$FeCl_3$	1	7.8 ± 1.1
Dithioerythritol	1	121.5 ± 2.1
β-Mercaptoethanol	1	124.1 ± 5.3
SDS	1	112.2 ± 2.1

To assess whether the PON arylesterase activities were directly related to Ca^{2+} ion, experiments were carried out that PON was inactivated for arylesterase activities. Because the arylesterase activity of PON is calcium dependent, each allozymic form was treated by either preincubation with 1 mmol/L EDTA for 0.5 h (resulting in a reduction of PON activity from 35 to 3 arylesterase units per mL) or removal of calcium ions with Chelex (activity was reduced to 0.8 arylesterase unit per milliliter). Under these conditions, paraoxonase activity was also essentially abolished.

Purified PON from human serum contains 3 cysteines; 2 of them form an intramolecular disulfide bond, while the third, at position 283, is free[23]. Cys283 was hypothesized to play a role in PON esterase activity, but earlier site-directed mutagenesis from this laboratory showed that substitution with either serine or alanine resulted in retention of PON arylesterase

activity[23]. In the current study, it was researched whether this plant PON enzyme's active site was the same or not. Reaction of the PON Cys283 with the sulfhydryl reagent PHMB caused a dose-dependent reduction in PON arylesterase activity, by 18, 54 or 95 % by using PHMB concentrations of 0.1, 1.0 or 10.0 mmol/L, respectively.

Iodoacetate, a smaller molecule than PHMB, was also used to block the PON free sulfhydryl group. On incubation of PON with 0, 1 or 10 mmol/L iodoacetate, PON arylesterase activities were 0.364, 0.287 or 0.287 U/mL, respectively. Even though PON arylesterase activity was minimally affected by this iodoacetate treatment.

In conclusion, paraoxonase is first purified from olive (*Olea europaea* L.) by using Sepharose 4B-L-tyrosine-1-naphthylamine affinity chromatography and some of its kinetic parameters have been investigated.

PON enzyme activity, taken as a diet with the antioxidant effects of plants was also determined. Atherosclerosis and vascular occlusion of these plants and heart disease, Alzheimer's disease, blood pressure, *etc.* positive effect on discomfort of the PON enzyme is determined in several studies. This is extremely important in terms of health of PON enzymes in oil seeds, especially in Turkey, widely used in the production of olive oil in the sixth identification, purification and characterization is extremely important.

The olive is of paramount importance to human nutrition as a physiologically important role in determining the existence of the paraoxonase enzyme and pure characterization is extremely important. Physiological importance of this enzyme paraoxonase, which has for the first time the existence of oil in seeds was determined by us in preliminary experiments. Purification and structure of this enzyme to clarify the future direction of studies related to the enzyme PON plant origin will be considered. A pioneer of this research is clear on this issue. Investigation of three-dimensional structure of the purified enzyme and the previously purified enzyme structure comparison with PON plant enzyme structure is most important in terms of lighting.

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