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EFFECTS OF FERULIC ACID ON HUMAN SERUM PARAOXONASE ENZYME PURIFIED BY THREE PHASE PARTITIONING

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Keywords
Paraoxonase 1,
Three-Phase Partitioning Method;
Ferulic Acid

Abstract: Human serum paraoxonase 1 (PON1) is a enzyme which inhibits macrophage cholesterol biosynthesis, metabolizes peroxides of cholesterol esters and reduces cholesterol efflux into macrophages. Therefore, it is speculated to play a role in several human diseases including diabetes mellitus and atherosclerosis. In this study, paraoxonase enzyme was first purified from human serum with three-phase partitioning method (TPP), also kinetic, characterization studies, and effects of ferulic acid were carried out. TPP purification process was performed in three stages. In first stage, PON1 was exposed to 60-80% ammonium sulfate precipitation, in the second stage, 1,0:0,5 ratio of human serum/ t-butanol and 20% ammonium sulfate saturation were used. In the third stage, the second TPP stage made over the intermediate, PON1 enzyme was purified from human serum with 49,87% recovery and 182,66 purification fold using constant ratio of human serum:t-butanol. In studies of the purified enzyme for kinetic properties, optimum pH, stable pH, optimum temperature, stable temperature were determined as 8,1, 7,0, 37°C, 20°C, respectively. Molecular weight of enzyme was found to be 45 kDa from Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). In addition, the inhibition effects of ferulic acid on the purified PON1 enzyme were investigated and Lineweaver Burk plots were obtained.

FERULİK ASİTİN ÜÇ FAZLI BÖLÜMLEMEYLE ARINDIRILMIŞ İNSAN SERUMU PARAOKSONAZ ENZİMİ ÜZERİNDEKİ ETKİLERİ

Anahtar Kelimeler
Paraoksonaz 1,
Üç fazlı ayrıştırma Yöntemi,
Ferulik asit

Özet: İnsan serum paraoksonaz 1 (PON1), makrofaj kolesterol biyosentezini inhibe eden, kolesterol esterlerinin peroksitlerini metabolize eden ve makrofajlara kolesterol akışını azaltan bir enzimdir. Bu nedenle, şeker hastalığı ve damar sertliği dahil olmak üzere birçok insan hastalığında rol oynadığı tahmin edilmektedir. Bu çalışmada paraoksonaz enzimi ilk kez insan serumundan üç fazlı ayrıştırma ile (TPP) saflaştırılmıştır, ayrıca kinetik karakterizasyon çalışmaları ve ferulik asidin etkileri çalışılmıştır. TPP saflaştırma prosesi, üç aşamada gerçekleştirilmiştir. Birinci aşamada PON1 %60-80 amonyum sülfat çökelmesine maruz bırakılmış, ikinci aşamada 1,0:0,5 oranında insan serumu/t-bütanol ve %20 amonyum sülfat doygunluğu kullanılmıştır. Ara ürün üzerinden yapılan ikinci TPP aşaması olan üçüncü aşamada, PON1 enzimi sabit insan serumu:t-butanol oranı kullanılarak insan serumundan %49,87 geri kazanım ve 182,66 kat saflaştırma ile elde edilmiştir. Saflaştırılan enzimin kinetik özellikleri için yapılan çalışmalarda optimum pH, stabil pH, optimum sıcaklık, stabil sıcaklık sırasıyla 8,1, 7,0, 37°C, 20°C olarak belirlenmiştir. Sodyum Dodesil Sülfat Poliakrilamid Jel Elektroforezinden

(SDS-PAGE) enzimin moleküler ağırlığı 45 kDa olarak bulunmuştur. Ayrıca, ferulik asidin saflaştırılmış PON1 enzimi üzerindeki inhibisyon etkileri araştırılmış ve Lineweaver Burk grafikleri elde edilmiştir.

1. Introduction

Paraoxonase (PON) is a calcium-dependent serum esterase, a member of the polygene family with PON1, PON2 and PON3, having both arylesterase (E.C. 3.1.1.2) and paraoxonase (E.C.3.1.8.1) activity. PON1 has a molecular weight of 43-45 kDa and is circulated bound to HDL [1]. The isoelectric point of PON1 is 5.1.PON1 enzyme has paraoxonase activity that provides hydrolysis of paraoxon, arylesterase activity that provides hydrolysis of phenylacetate, and lactonase activity that provides hydrolysis of lactones [2-4].

It is known that paraoxonase plays an important role in the prevention of atherosclerosis and drug metabolism in recent years [5-6]. Paraoxonase has a protective effect against atherosclerosis by both preventing the oxidation of LDL and HDL lipids and metabolizing lipid peroxides thanks to its lactonase activity [7-8]. It has been determined that PON1 enzyme level and activity decrease in cardiovascular diseases, hypercholesterolemia patients, elderly people, obesity, menopause, diabetes, Parkinson's disease and kidney failure [9-11].

Precipitation, chromatographic methods, dialysis and filtration methods are prominent as enzyme purification methods. However, these methods include processes with many steps, loss of time, difficulty in implementation, and loss of high efficiency. Three-phase partitioning (TPP) is an easy-to-use method that has come to the fore in recent years for the purification of proteins. With TPP, it is possible to perform bioseparation of two or more compounds by single-step extraction. Taking advantage of the different physicochemical properties of the three liquid layers, the separation of these systems with a single extraction is one of the benefits of this system. TPP method is a technique that can use salting out, isoionic precipitation, precipitation with co-solvent, osmolytic precipitation and cosmotropic precipitation techniques together. In TPP, t-butanol is used to precipitate proteins, form triple-phase layers and remove lipids, phenolic compounds, and some detergents [12-17].

2.1. Materials

Human blood serum was obtained from Atatürk University Research Hospital Biochemistry Laboratory, Erzurum, Turkey. Ammonium sulfate, t-butanol, coomassie Brillant Blue R-250 and other chemicals, reagents were purchased from Sigma Chem. Co.

2.2. Determination of paraoxonase

500 µL substrate (1 mM paraoxon), 400 µL buffer (50 mM Tris/HCI buffer containing 1 mM CaCI2 pH 8.0) and 100 µL purified enzyme were taken a test tube (molar extinction coefficient ε: 18,000 M-1 cm-1, pH 8.0). The reaction was incubated at 37 °C for 15 min. Then, it was measured spectrophotometrically at 412 nm. Measuring the activity is based on absorbance of paranitrophenol at as a result of the reaction between paraoxon and PON1. The enzyme unit of paraoxonase is the quantity of micromoles of the paraoxon hydrolyzed per min. Pure water was used as blank [18-19].

2.3. Protein Concentration

Concentration of protein was determined by Bradford method using Coomassie brillant blue G-250 dye as reagent by measuring the absorbance at 595 nm at room temperature. 5 mL Comassie brillant solution and 10 µL purified enzyme were taken a test tube, after vortex and incubation 25 °C for 10 min, it was measured spectrophotometrically at 595 nm. Pure water was used as blank [20].

2.4. Three–Phase Partitioning (TPP)

2.4.1. Effect of t-butanol

TPP experiments were performed by applying various t-butanol ratios (human blood serum: t-butanol; 1.0:0.5, 1.0:1.0, 1.0:1.5, 1.0:2.0) with a constant ammonium sulfate saturation at 20% (w/v). The mixture was mixed in magnetic stirrer for 30 min at room temperature. Then, it was centrifuged at 5000 rpm for 10 min at +4°C to facilitate the seperation of phases. The bottom phase and the interfacial phase were separately collected and dialyzed against 50 mM Tris/HCI buffer containing 1 mM CaCI2 pH 8.0 for 3 hours (Figure 1). Each of phases was analyzed for enzyme activity and protein content. The experimental conditions at which the highest enzyme activity observed were selected for the further experiments [21].

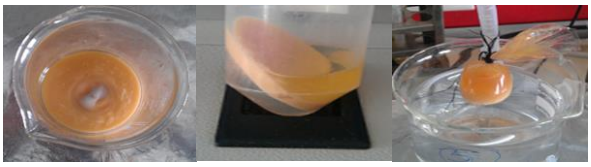

Figure 1. Steps of purification of paraoxonase by TPP

2.4.2. Effect of Ammonium Sulfate Saturation

The effects of different ammonium sulfate saturations (20, 40, 60, 80%) (w/v) were performed

at the best recovery activity crude enzyme:t-butanol ratio is obtained first step in TPP. The interfacial phase were collected and dialyzed against 50 mM Tris/HCI buffer containing 1 mM CaCI2 pH 8.0 for 3 hours. Each of phases was analyzed for enzyme activity and protein content. The experimental conditions at which the highest enzyme activity observed were selected for the further experiments [21].

2.4.3. Optimization of TPP conditions

For the second step of TPP, the highest activity recovery of aqueous phase from the first step was used for optimization of TPP conditions. Selected phase wasn't dialyzed. t-butanol in ratio of 1.0:0.5 was added, then saturated with different ammonium sulfate to final concentration of 25, 30, 35, 40, 45, 50%. The enzyme recovery and protein content of phases were analyzed as previously mentioned [21].

2.5. SDS-PAGE

Molecular weight of purified paraoxonase (PON1) was determined by discontinuous SDS-PAGE according to the method of Laemmli on a Biorad Mini Protean electrophoresis [22]. Electrophoresis was performed at 100 mV-160 mV for 5 hours in 3% upsetting gel and 10% separating gel. For each lane 40 µL enzyme was applied. The gel was stained Coomassie Brillant Blue R-250 for 45 min then destained by 30% methanol and 10% acetic acid for 2 hours [23].

2.6. Kinetic Studies

In order to determine the optimum pH value of the PON1 enzyme, the enzyme was kept in a water bath at 37°C for 15 minutes in appropriate buffers for different pH values (Acetate buffer (10 mM) for values of pH 3.0, 4.0, 5.0; Tris/HCl buffer (10 mM) for values of pH 6.0, 7.0, 8.0; Glycine buffer (10 mM) was used for values of pH 9.0, 10.0.), and then its activity was determined spectrophotometrically at 279 nm [24].

To determine the stable pH of the enzyme, using the corresponding buffers given in the above paragraph at varying pH values (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) The prepared samples were stored at +4°C for 7 days. Samples were prepared using 500 µL of buffer used for the relevant pH value and 500 µL of enzyme. 100 µL of these samples, which were prepared in the same time zone for each day, was taken and 1.4 mL of substrate was added. Then, after 15 minutes in a 37°C water bath, spectrophotometric measurements were made at 279 nm [24].

In order to determine the optimum and stable temperature value of the enzyme, the prepared samples were kept at different temperatures (10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C) for 15 minutes and 1 hour, respectively, and then the activity was determined at 279 nm. Samples were prepared to contain 100 µL of buffer + enzyme and 1.4 mL of substrate. Pure water was used as the blank [24].

2.7. In vitro Study for Ferulic Acid

10 mM, 50 mL stock solutions of ferulic acid were prepared. Samples were prepared Using 50 µL of enzyme and varying amounts of ferulic acid, 500 µL of 10 mM Tris/HCl buffer (pH 8.0) containing 1 mM CaCl2, 300 µL of paraoxane solution dissolved in 2.5 mL acetone and prepared at a concentration of 3 mM with a final volume of 1 mL. Spectrophotometric activity was determined at 412 nm using paraoxane substrate in the samples, which were kept in a water bath at 37°C for 15 minutes.

Based on the measurements taken, %Activity-[I] plots for ferulic acid were obtained and IC50 values were calculated using the curve equations. In order to determine the Ki values of these compounds, 3 different inhibitor concentrations were determined by using the concentration of the compounds that halved the purified PON1 enzyme activity. Stock solutions of the related compounds were prepared at these three different concentrations. Samples was prepared using 500 µL of 10 mM Tris/HCl buffer (pH 8.0), 50 µL of enzyme, varying values (50 µL, 90 µL, 140 µL, 190 µL, 230 µL) of 3 mM paraoxane, varying amounts of distilled water and antioxidant compounds (Final volume is 1 mL). Afterwards, the samples were kept in a water bath at 37°C for 15 minutes and measured spectrophotometrically at 412 nm. Lineweaver-Burk graphs were drawn for inhibitors with the obtained data [25].

3. Results and Discussions

3.1. Results of Paraoxonase Enzyme Purification Steps

According to the volume of human serum used in the purification of the paraoxonase enzyme, n-butanol was used at the ratios of 1.0:0.5, 1.0:1.0, 1.0:1.5, 1.0:2.0. In the activity measurements, the ratio of n-butanol giving the maximum activity value (51.89) for the paraoxonase enzyme was 1.0:0.5 as seen in Figure 2.

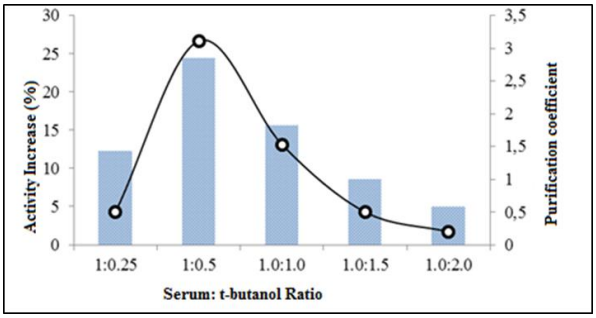

Figure 2. n-butanol optimization in PON1 enzyme purification studies

In ammonium sulphate optimization studies, 20% ammonium sulphate rate was determined as the most appropriate rate (Figure 3). The sample with 20% ammonium sulphate rate was

again subjected to the 2nd ammonium sulphate optimization. Here, the best activity gain was obtained at 25% ammonium sulfate saturation (Figure 4).

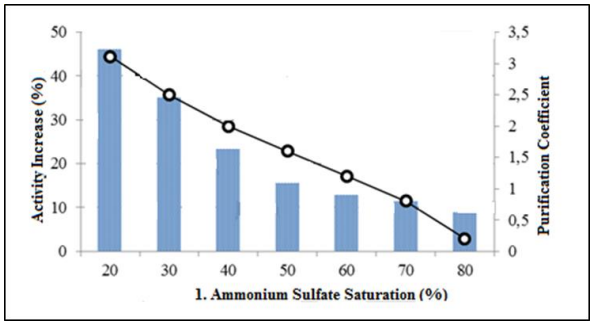

Figure 3. 1. Ammonium sulfate optimization in PON1 enzyme purification studies

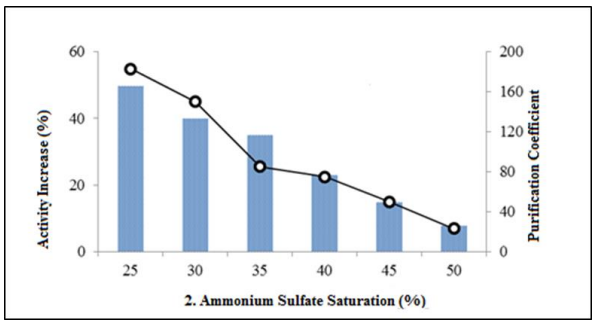

Figure 4. 2. Ammonium sulfate optimization in PON1 enzyme purification studies

In the purification stages of the paraoxonase enzyme with TPP, both activity and protein determination were made. With the data obtained as a result of the measurements, parameters such as activity, total activity, specific activity, purification coefficient, % yield were calculated. Calculated parameters of PON1 purified with TPP were given in the Table 1.4.

Table 1. Purification and yield profile of human serum PON1 purified by TPP

Steps	Volume (mL)	Activity (EU/	Total Activity (EU/mL)	%
Serum	50	821,42	$4,11.10^4$	-----
A. Sulphate (%60-80)	15	1216,22	$1,82.10^4$	24,42
1.TPP	15	1266,69	$1,9.10^4$	46,23
2.TPP	15	1368,63	$2,05.10^4$	49,87

Steps	Specific Activity (EU/mL)	Protein (mg protein / mL)	Purification Coefficient
Serum	0,088	$9,32.10^3$	------
A. Sulphate (%60-80)	2,85	427	2,53
1.TPP	7,24	175	3,11
2.TPP	575,8	2,377	182,66

PON1 enzyme was purified from human serum from 60-80% by ammonium sulfate precipitation and hydroponic interaction chromatography methods [26]. In enzyme activity measurements made at 60-80% ammonium sulfate precipitation, 83,588 U/mL activity, 1,504,58 U total activity, 0.0783 U/mg specific activity, 45.37% yield and 1.74 purification coefficient were obtained. By hydrophobic interaction chromatography, 131,404 U/mL activity, 394.21 U total activity, 11.76 U/mg specific activity, 11.89% yield and 150.19 purification coefficient were obtained. According to the results obtained in this study (see as Table 1), the purification method with the TPP method stands out as an advantage over other methods.

3.2. SDS-PAGE Image of Human Serum Paraoxonase Enzyme

In the purification studies for the PON1 enzyme from human serum, values between 43-45 kDa were found in molecular weight determination [27-30]. The molecular weight of the PON1 was determined as 40-45 kDa [31-32]. Our data related to molecular weight of the PON1 was determined as 45 kDa (Figure 5). These results support each other.

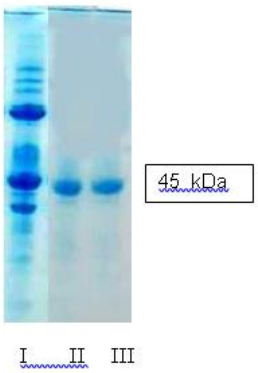

Figure 5. SDS-PAGE image of paraoxonase enzyme purified by TPP. I: standard protein myosin (200 kDa), β-galactosidase (125 kDa), bovine serum albumin (BSA) (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), lysozyme (14.3 kDa) II and III purified PON1 enzyme 45 kDa.

3.4. Results of Kinetic Studies

According to the data obtained, graphs of optimum pH in Figure 6, optimum temperature in Figure 7, stable pH in Figure 8, and stable temperature in Figure 9 were given.

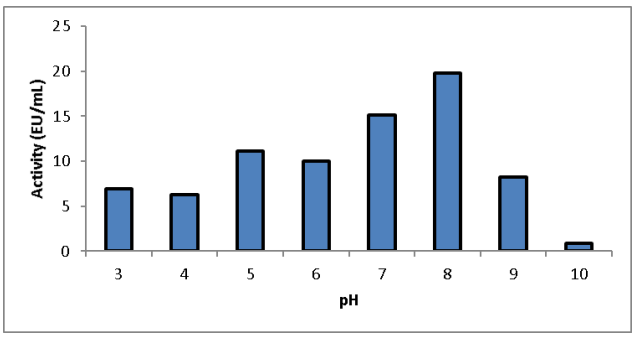

Figure 6. Optimum pH graph obtained in PON1 enzyme purification studies

According to the data obtained in optimum pH studies, the highest enzyme activity (19.8%) was obtained at pH 8.1. In a study conducted by Kanamori-Kataoka and Seto in 2009, they measured the activity at pH 8.0 [30]. In the study conducted by Sayın and Özensoy Güler (2015), bovine serum paraoxonase enzyme was purified and the variation of the activity with pH was investigated [33]. The highest % activity value for bovine serum paraoxonase enzyme at pH 8.0 was found in this study. In the study published by Demir et al. (2008), the optimum pH value of the PON1 enzyme purified from human serum was expressed as 8.0 [29]. However, in the study conducted by Aşkın et al. (2012), it was stated that this enzyme purified from beef liver has a high activity in the pH range of 7.0-7.5 and shows its maximum activity at pH 7.1 in this range [34].

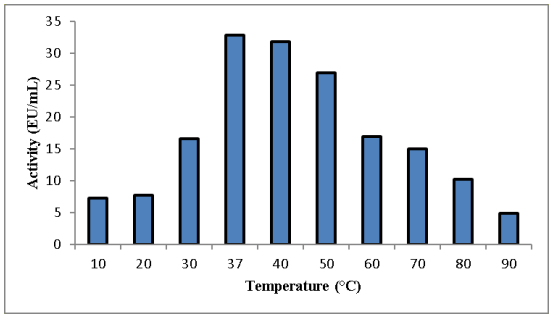

Figure 7. Optimum temperature graph obtained in PON1 enzyme purification studies

Rodrigo et al. (2001) made the highest paraoxonase enzyme activity measurements at pH 8.5 and 37°C. According to the data obtained from the optimum temperature studies of the purified enzyme, the highest activity value was obtained at 37°C with 32.81%. An activity measurement of 31.85% was made at 40°C, which is close to this value. However, at temperatures below and above these temperatures, significant decreases were observed in the % activity value of the enzyme. A decrease in activity is expected, as the enzyme is prone to denaturation with temperature due to its protein structure above 40°C [35]. In the study published by Aşkın et al. (2012), some kinetic properties of the paraoxonase enzyme were examined. In this study, the activity of the PON1 enzyme reached the highest value with an average absorbance of 2,228 at 37°C [34]. These values are in agreement with the data obtained in our study.

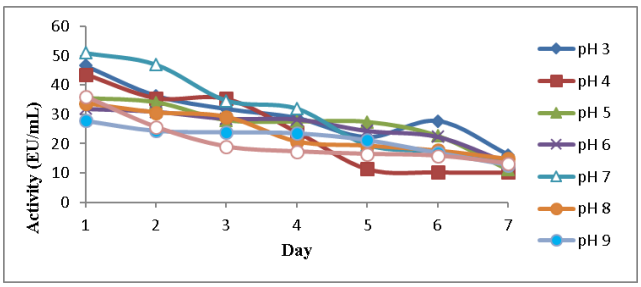

Figure 8. Stable pH graph obtained in PON1 enzyme purification studies

According to the data obtained in stable pH studies, enzyme activity decreased for 7 days compared to the first day at all ambient pHs. On the 1st day it was the pH 7.0 medium that gave the highest activity (50.94), while on the 7th day it was the pH 8.0 medium that gave the highest activity (14.79). The pH 7.0 medium was accepted as the stable pH value for the purified paraoxonase enzyme.

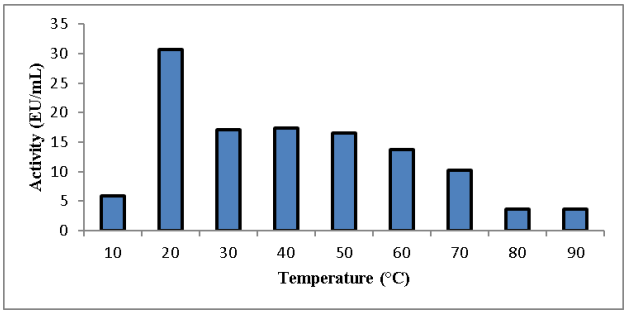

Figure 9. Stable temperature graph obtained in PON1 enzyme purification studies

In the stable temperature studies carried out for the purified enzyme, a temperature environment of 20°C was accepted as a stable temperature.

3.3. in vitro Study of Ferulic Acid on Purified Paraoxonase Enzyme

The graph used to determine the IC50 values of ferulic acid was given in the Figure 10.

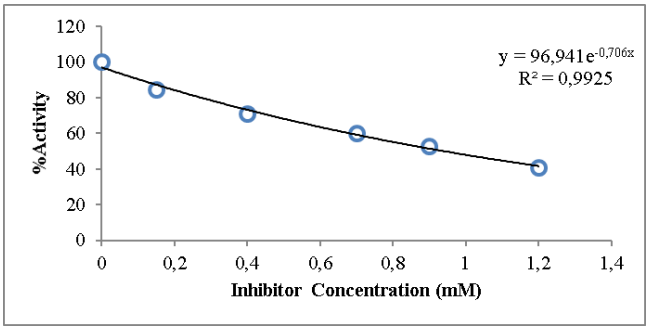

Figure 10. Effect of ferulic acid on human serum PON1 enzyme activity

Lineweaver-Burk graphs were drawn to determine the Ki values and inhibition type of the purified

human serum PON1 enzyme for ferulic acid substrates (Figure 11). Ki values and inhibition type were determined for the substrates by using these graphs [36]. In the calculation of Ki value, the formula $K_{M^I} = \frac{K_M I}{\left(1+\frac{[I]}{K_i}\right)}$ was used in competitive inhibition types, while the formula

$V_{Max^I} = \frac{V_{Max}}{\left(1+\frac{[I]}{K_i}\right)}$ was used in non-competitive inhibition types.

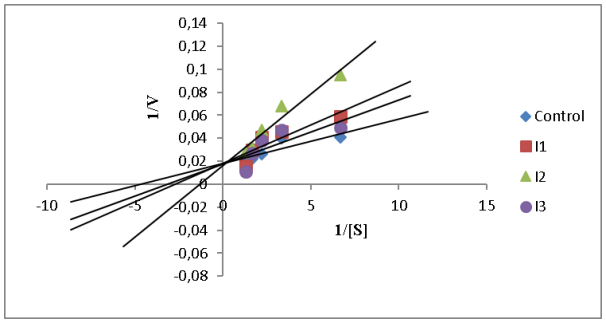

Figure 11. Lineweaver-Burk graph of ferulic acid for human serum PON1 enzyme

The IC50 value for ferulic acid was 0.938 mM, Ki values were 0.370, 0.770, 0.314, Ki mean value was 0.485, inhibition type was determined as competitive.

Conclusions

Using the TPP method, PON1 enzyme was purified from human serum in a shorter time and with higher efficiency, and the inhibition effect of ferulic acid was investigated. The activity recovery of the enzyme was found to be higher than the purification studies of the PON1 enzyme by some chromatographic methods. The fact that the purification steps are fast, cheap and can be done in three steps, especially besides the high activity gain, shows the potential of this process and has a great advantage for industrial-scale enzyme purification applications.

Conflict of Interest

The author declare that she has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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