Impact of some metal ions on activity of purified human enzyme paraoxonase (hPON1)

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**ABSTRACT**

Inhibition of enzymes has a great influence on various metabolism activity in the body. The objective of this research has been to assess the impact of some metals and the drug statin on the activity of purified enzyme paraoxonase extracted from human sera (hPON1). The enzyme was purified in consecutive four steps using Ammonium Sulphate, Dialysis, Ion exchange and Gel filtration chromatography, respectively. These four purification steps have approved their credence in obtaining a maximum purification state of the enzyme. The inhibitory impact of some metals ions, i.e. Mg+2, K+1, Mn+2, Na+1, EDTA, and Zn+2, on the activity of purified hPON1 has also been assessed. The result showed the highest inhibitory impact of 10 mMol/L of Sodium Nitrate (NaNO3) on the activity of purified hPON1 while medication atorvastatin had significantly increased the activity of hPON1. It is concluded that any subtle change in the enzyme PON-1 could consequently lead to various diseases. More assessments are required for type two (PON-2) and type three (PON-3) to be explored. Atorvastatin is recommended to regulate cholesterol metabolism by the intestine and to lower the risk of diseases for elder ages.

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**INTRODUCTION**

The enzyme paraoxonase (PON-1) has been identified and classified by Abraham Mazur and Norman Aldridge since the middle 1940s to early 1950s (Aldridge, 1953). Initially, the enzymes were referred to as “A”-esterases, but later became universally known as paraoxonase due to their ability to detoxify the organophosphate compound paraoxon which is the toxic metabolite of parathion, a commonly used agricultural insecticide (Mackness and Mackness, 2015). In the early 1960s and 1970s studies demonstrated that PON1 activity was polymorphically distributed in human populations and the frequency of the low activity phenotype varied among populations of different ethnic origins (von Mallinckrodt and Diepgen, 1988). The enzyme PON1 is the first discovered member of a multigene family also containing PON2 and PON3, the genes for which are located adjacent to each other on chromosome (Harel et al., 2004). The human serum paraoxonase-1 (hPON1) is a calcium-dependent hydrolytic enzyme that is found in a variety of mammalian species. It is a high-density lipoprotein- (HDL) associated serum enzyme that exhibits a broad substrate specificity, i.e. speculated to play a role in several human diseases including diabetes mellitus and atherosclerosis (Furlong et al., 2010). While the serum PON-1 is found in all mammalian species studied so far but not in sera of birds, fish and reptiles nor in insects (Shunmoogam et al., 2018). In addition to protecting property against exposure to some organophosphorus (OP)
pesticides by hydrolyzing their toxic oxon metabolites, PON1 is important in protecting against vascular disease by metabolizing oxidized lipids (Furlong et al., 2010).

The hPON1 is synthesized in the liver and is secreted into serum as an HDL-associated protein (Kowalska et al., 2015). As an antioxidant enzyme carried by HDL, it can hydrolyze lipid peroxide in lipoproteins as a result of its lipolactonase activity (Berrougui et al., 2012). This is done by decreasing oxidative stress via decreasing oxidative stress in serum lipoprotein, macrophages and atherosclerotic lesions (Yang et al., 2017). The hPON1 could partly be responsible for the anti-inflammatory and anti-atherogenic properties of HDL molecules in Blood (Eren et al., 2012). Low hPON1 activity has been associated with an increased risk of major cardiovascular events. Therefore a variety of studies had already established the cardio-protective properties and clinical relevance of hPON1 (Shunmoogam et al., 2018).

The objective of the current study has been to explore our understanding of the impact of some metal ions and specific medication, e.g. atorvastatin on the activity of purified PON1 in human (hPON1).

MATERIALS AND METHODS

The materials used contained DEAE-Cellulose Sephadex G50 fast flow (Sephadex G100) Paraoxon from Sigma Company. Collection of fresh sample sera of (50 ml) from human blood were done from the Central Blood Bank (CBB) of Nineva City from individuals aged 30 years with no medication consumed, nonsmoker healthy men.

Assay of PON1 activity
Paraoxonase-1 activity was determined by using method described by (Mastorikou et al., 2008). A 10 mL of serum was added to the Tris buffer, (Tris-HCl of 50 mMol, pH8) contained 20mMol of CaCl₂ and 5mMol/L of Paraoxon (Sigma). Initial rate of hydrolysis was determined by liberating P-nitro Phenol via 410nm at 25°C. The enzyme activity was expressed in international units per 1 min of serum (U/min) by using the standard curve of P-nitro Phenol.

Determination total protein in sample
Absorbance at 280 nm was used to monitor the protein in the column effluents and ammonium Sulphate precipitation. Protein was the determination by Quantitative measurements at 560 nm according to modified Lowy and Pollak method, 1973 (Schacterle and Pollack, 1973) while the standard curve of bovine serum albumin was determined using an extinction coefficient of 0.67 (Holm and Peck, 1988).

Purification of Enzyme paraoxonase (PON1)
50 ml of human serum was collected from healthy human blood, centrifuged at 2000/rpm for 10 min. The hPON1 was then precipitated by adding a small amount of 70% Ammonium Sulphate to dissolve using magnetic stirred electrically at (4°C) for 60 min and the samples were then left overnight in the refrigerator. The precipitate was separated by centrifugation at 6,000xg for 20 minutes in cool centrifuge followed by determining the protein concentration and PON1 activity. The dialysis of Paraoxonase precipitate was placed in a large volume of Ammonium bicarbonate and stirred with magnetic stirrers overnight at (4°C). The paraoxonase preparation was lyophilized and the powder was stored in deep freeze. The ion exchange, including DEAE-Cellulose Sephadex, G50 fast flow and Sephadex G-100 purification of the enzyme was achieved using the method of (Sinan et al., 2006). The column was equilibrated with (0.1M) Tris-HCL buffer of pH8 containing 15ml of enzyme solution. The paraoxonase was then eluted with linear of (0.1-1M) of NaCl in Tris-HCL buffer pH8. The purified PON1 was stored at (4°C).

Sodium Dodecyl Sulphate Polyacrylamide Gel-electrophoresis (SDS-PAGE)
The SDS-PAGE was performed after purification of the enzyme using 5% and 3% acrylamide concentrations, contained 10% SDS, for the running and stacking gel, respectively (Laemelli, 1970).

Inhibition and activation of purified enzyme paraoxonase
To assess the inhibitory effects three different concentration of ion metals Mg²⁺, K⁺, Mn²⁺, Na⁺, EDTA, and Zn²⁺, (1, 5 and 10 mMol/ml) were added to the purified enzyme paraoxonase activity, while no metal was added as 100% activity, used as con-
Table 1: Summary of Purification of human PON1

| Fraction                          | Volume (ml) | Activity μ/ml | Total activity μ/ml | Protein amount mg/ml | Total protein mg/ml | Specific activity mg/μl | Over load yield% | Over all purification (fold) |
|----------------------------------|-------------|---------------|---------------------|----------------------|----------------------|--------------------------|-----------------|-------------------------------|
| Serum                            | 50          | 150           | 7,500               | 1.4                  | 70                   | 107.1                    | 100             | 1                             |
| Ammonium Sulphate Precipitation  | 31          | 200           | 6,200               | 1.1                  | 33                   | 187.8                    | 82.6            | 1.75                          |
| Dialysis                         | 23          | 185           | 4,255               | 0.8                  | 18.4                 | 194.2                    | 47.6            | 1.81                          |
| Ion exchange DEAE-Cellulose      | 15          | 90            | 1,350               | 0.2                  | 3                    | 450                      | 18              | 4.2                           |
| Gel Filtration Sphadex G100      | 9           | 77            | 693                 | 0.09                 | 0.81                 | 855                      | 9.24            | 7.98                          |

Table 2: Three different concentrations (1, 5 and 10 mMol/ml) of the 7 metal ions added to the serum to demonstrate their inhibitory impacts on activity of hPON1.

| Compound       | Enzyme activity (U/min) | Activity yield of original (U/min) |
|----------------|-------------------------|-----------------------------------|
| Standard       | 30                      | 100                               |
| MgCl₂ (1mM/mL) | 20                      | 66.7                              |
| MgCl₂ (5mM/mL) | 21                      | 63.3                              |
| MgCl₂ (10mM/mL)| 19                      | 83.3                              |
| KCN (1mM/mL)   | 18                      | 66.7                              |
| KCN (5mM/mL)   | 25                      | 56.5                              |
| KCN (10mM/mL)  | 20                      | 73.3                              |
| MnCl₂ (1mM/mL) | 17                      | 56.5                              |
| MnCl₂ (5mM/mL) | 22                      | 50                                |
| MnCl₂ (10mM/mL)| 17                      | 33.3                              |
| NaNO₃ (1mM/mL) | 15                      | 33.3                              |
| NaNO₃ (5mM/mL) | 10                      | 30                                |
| NaNO₃ (10mM/mL)| 9                       | 30                                |
| Na₂SO₄ (1mM/mL)| 16                      | 53.3                              |
| Na₂SO₄ (5mM/mL)| 20                      | 66.7                              |
| Na₂SO₄ (10mM/mL)| 11                     | 36.6                              |
| EDTA (1mM/mL)  | 15                      | 50                                |
| EDTA (5mM/mL)  | 17                      | 63.3                              |
| EDTA (10mM/mL) | 12                      | 40                                |
| ZnSO₄ (1mM/mL) | 19                      | 63.3                              |
| ZnSO₄ (5mM/mL) | 18                      | 60                                |
| ZnSO₄ (10mM/mL)| 12                      | 50                                |
Table 3: Assessment of activity of Purified PON1 by Using atorvastatin (20mg)

| Standard of purified PON1 | Activity | Yield of original activity |
|---------------------------|----------|---------------------------|
| hPON1/Activity            | 30       | 100                       |
| 2.0 mM/m/drug             | 32       | 106.6                     |
| 4.0 mM/m/drug             | 34       | 113.3                     |
| 6.0 mM/m/drug             | 35       | 113.3                     |
| 10.0 mM/m/drug            | 40       | 133.3                     |
| 12.0 mM/m/drug            | 40       | 133.3                     |

trol. Meanwhile, to assess the inhibitory effects of metal ions on the activity of purified PON1 20mg of atorvastatin as an activator was added to six different concentrations of hPON1.

RESULTS AND DISCUSSION

The 50 ml serum has undergone consecutive left-over purification using four different techniques in which its volume declined following each purifying stage (Table 1). The final product of the purified hPON1 obtained using Gel filtration Sphadex G100 was 9.24% denoting, almost eightfold purity in comparison with the initial stage. Accordingly, the specific activity of hPON1 was increased up to 855 mg/u/ml. Simultaneously, the total protein was decreased following each step down to 0.81 mg/ml.

The inhibitory impacts of the seven metal ions on the activity of the hPON1 using three different concentration (1, 5, 10 mM/ml) had produced variable results. While the general impact of these metals considerably inhibited the yield of enzyme hPON1 the highest inhibiting effects were measurable at the lowest concentration (1 and 5 uM/ml) in some ion metals, i.e. NaNO$_3$ while others varied with other two higher concentrations (5 and 10 uM/ml) (Table 2). The Line weaver-Burk Plot had confirmed the maximum inhibition of 1uM/ml of NaNO$_3$ (Figure 1). The results showed that Vmax remained constant while Km increased with increasing the concentration of inhibitor predating that NaNO$_3$ acted as a non-competitive inhibitor.

The addition of 20mg of artovastin to hPON1 enzyme had increased the yield of original activity to a maximum 113% mg/u/ml at a dose 4.0mMol but showed no effects then after (Table 3).

Paraoxonases, with three isoenzymes are a family of mammalian enzymes with aryl-dialkyl-phosphatase activity. They were originally described for their involvement in the hydrolysis of organophosphates while their enzymatic activity is more diversified than activity as an organo-phosphatase (Bergmeier et al., 2004). Most of the studies on the paraoxonase family have specifically looked at the paraoxonase type-1, leaving much to be learned about the remaining two (Garelnabi et al., 2012). In the current study, the hPON-1 has been purified in four consecutive stages to a yield 9.24% purification which represented an almost eightfold purity in comparison with the initial stage. The latter implies the credence of these purification steps to guarantee a higher purity in comparison with other studies. The purified enzyme could then after be used for further experiments which produce significantly reliable results.

Inhibition of the enzyme is an undesirable process and might be very dangerous to the body, i.e. any changes in PON1- enzyme activity, by drug admission or/and any chemicals taken in, i.e. the activity of an enzyme can be decreased by the non-covalent binding of inhibitors. The latter is an important issue because many drugs function as enzyme inhibitors (Robyt et al., 1987). Many investigators have noted the inhibitory impact of some chemical compounds on paraoxonase (PON-1-) activity leading to many potential consequences in preventative medicine and toxicology as well as in certain societal contexts (Sutherland et al., 1995). The genes that encode for these iso-enzymes have a number of different polymorphisms, which created additional interest in the study of this enzyme group and its potential ethnic variations (Costa and Furlong, 2002). Additional research on the inhibition and selective inhibition, specifically of PON1, has been done to shed some light on the connections between decreases in enzymatic activity of individuals with cardiovascular diseases (Nguyen and Sok, 2003).

The role of enzyme PON-1 is to protect the high-density protein (HDL) from the low oxidation density of lipids (LDL) it provides. Any decrease in paroxonase activity may be associated with a decrease in the levels of HDL, as this enzyme is associated with HDL. Consequently, decreased activity of paroxonase and increased of LDL in patients with elevated cholesterol (triglycerides) could lead to atherosclerosis, making them predisposed to early
coronary artery disease. Evidence also refers that this family of enzymes has some role in the human innate immune system (Ozer et al., 2005).

Many researchers have shown inhibition of PON-1 with heavy metal ions, i.e. the protective activity and the body levels of this calcium-dependent ester hydrolase can be affected by different factors (Levi and James, 2000). Based on some in vitro and in vivo experiments, higher concentrations of lead (Pb^{2+}) in the blood (<1 μg/dl), can cause a remarkable inhibition in PON1 activity Li et al. (2006). Additionally, due to the protective role of PON1 against atherosclerosis, such metal caused inhibition, could finally affect the occurrence rate of atherosclerosis among populations (Kamal et al., 2011). Inhibitory impacts of some heavy metal (Pb^{2+}, Cr^{6+}, Fe^{3+} and Zn^{2+}) on purified PON-1 lead to decrease on PON-1 activity at low concentration of metals. Also in the researcher (Ekinci and Beydemir, 2010) and metal ions such as Co, Cu, Mn, Hg on PON-1 activity in Rat liver lead to decreased of PON-1 activity, this is due to binding these metals to active sites in protein PON1 (Pla et al., 2007). The impact of seven metal ions using three different concentration (1, 5, 10 mM/ml) had produced variable inhibitory results via lowering the yield of enzyme hPON1. These results are in consistence with the work of (Ekinci and Beydemir, 2010) as the activity of PON-1 decreased due effects of all the metals being used. The highest inhibiting effects were measured has been in the lowest concentration (1 and 5 uM/ml), i.e. NaNO₃. The latter might represent the most effective and sensitive metal ions against the activity of PON-1 while others varied pending with other two higher concentrations (5 and 10 uM/ml). This result is inconsistent with many other works (Sayin et al., 2012).

Atorvastatin belongs to a group of medicines known as a statin, are lipid (fat) regulating medicine which lowers lipids known as cholesterol and triglycerides in the blood when a low-fat diet and lifestyle changes on their own have failed. Many blood lipid medications can inhibit the PON1 activity which in turn increases LDL metabolism, leading to increase PON1 mRNA levels, which reduces food cholesterol absorption from the small intestine (Hernández et al., 2009).

The addition of 20mg of atorvastatin to hPON1 enzyme had increased the yield of original activity to a maximum 113 mg/u/ml at a dose 4.0mMol but showed no effects then after. Few studies have analyzed that potential effect of statins on purified serum PON-1, from a normal human, but were restricted to the analysis of activity measurements from an in vitro study (Aviram et al., 1998). The increase mechanisms of the purified PON-1-activity in a dose- depended on the manner with a maximum effect at (12 mM/ml) of atorvastatin produced compatible results contrast with another study proposing the positive impacts of statine on PON-1 activity (Gouédard et al., 2003). Different concentrations of atorvastatin and the first activity of the purified enzyme was revealed the effectiveness of the enzyme. This can be attributed to the fact that the enzyme is mainly present in the blood linked with the high-density protein and that it has the ability to prevent oxidation of the high-density protein. Therefore, the protective effect of HDL associated paraoxonase confirmed the inhibitory action over the biological activity of minimally oxidized LDL (Watson et al., 1995).

Our results had clarified the efficacy of the purified paraoxonase enzyme, which increased with an increase in the concentration of atrovastatin. This medication leads to lower cholesterol rate in the body and thus lead to a higher activity of Paroxone enzyme. Atorvastatin does metabolite in the intestine could, therefore be a potent antioxidants against lipoprotein oxidation (Kennedy et al., 2017).

CONCLUSIONS

The concentration of 0.35μg/ml may represent the highest efficacy of the purified enzyme from the healthy person’s serum. Different metal ions have different inhibiting impacts on the activity of purified human enzyme paraoxonase. Atorvastatin can regulate cholesterol metabolism by the intestine and lowers the risk of diseases for elder ages. The outcome might therefore be a pioneered in demonstrating the possibility of concomitant effectiveness of the PON-1 enzyme with the presence of atorvastatin.

Ethical approval and Acknowledgement

The nature of the research has been proposed to serve the public health where the research was carried out within the hospital of Ibn-Sina itself without dispute. The authors acknowledge their generous facilities in providing us with anonymous data on CKD.

Conflict of Interest

There also is no conflict of interest between other Governmental bodies nor between co-authors.

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