

Comparison of Serum Paraoxonase 1 (PON1) Activities Among Different Sheep Breeds in Turkey

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Abstract: Paraoxonase 1 (PON1, EC 3.1.8.1) is a calcium dependent mammalian enzyme that is synthesized primarily in the liver and is secreted into the serum where it is associated with High Density Lipoproteins (HDLs) and has a protective effect against oxidation of Low Density Lipoproteins (HDLs). Beside antioxidant and antiatherogenic properties, PON1 is also a detoxifier that can hydrolyze toxic organophosphates. Several studies have shown that PON1 can bind reversibly to organophosphate substrates which it hydrolyzes. Therefore, PON1 is the main means of protection of the nervous system against the neurotoxicity of organophosphates entering the circulation. This study was conducted to characterization of serum Paraoxonase 1 (PON1) activity from different sheep breeds namely Karacabey Merino, Kivircik, Tahirova, Akkaraman and Daglic. K_M and V_{max} values of five different sheep breed were determined by Lineweaver-Burk method. The values of V_{max}/K_M showed that Kivircik breed has the greatest PON1 activity, on the other hand, Karacabey Merino breed showed the least activity toward paraoxon substrate.

Key words: Sheep paraoxonase 1, Karacabey Merino, Kivircik, Tahirova, Akkaraman, Daglic

INTRODUCTION

Organophosphorus (OP) compounds include highly toxic pesticides and military nerve agents. Pesticides are extensively used all over the world and in recent years, their use has increased. Large amounts of these chemicals are released in to the environment and many of them affect non-target organism. The widespread use of pesticides in agriculture, public health and household environments results in continuous exposure of human populations (Lopez *et al.*, 2007). Organophosphates (OPs) are an important class of insecticides that have been widely used for treating sheep for ectoparasites as well as in other sectors of the farming industry (Povey, 2010). Health problems associated with acute OP toxicity are well defined but ill-health induced by chronic exposures to OPs remains controversial (Povey, 2010).

Different pesticides, including OPs have been reported to induce oxidative stress due to generation of free radicals and alteration in antioxidant defence mechanisms (Lopez *et al.*, 2007). Examining exposures to mixtures of pesticides is important for understanding the

underlying mechanisms of OP compound toxicity and assessing aggregate risk (Jansen *et al.*, 2009). Numerous studies have examined additive and synergistic effects of combinations of Ops *in vitro* and *in vivo* (Jansen *et al.*, 2009). The importance of the HDL-associated enzyme paraoxonase 1 (PON1) in OP detoxication has been known for some time (Costa, 2006). A number of OP insecticides are metabolised by microsomal oxidases to oxygen analogs, the active neurotoxic metabolites which are potent inhibitors of cholinesterases (Akğur *et al.*, 2003). The oxygen analogs are hydrolyzed by the serum A-esterase, Paraoxonase 1 (PON1) which appears to play a central role in their detoxication and in their toxicity (Akğur *et al.*, 2003). PON1 (EC 3.1.8.1) is a calcium dependent serum esterase that is synthesized by the liver. In serum, It is closely associated with high density lipoproteins (Gaidukov and Tawfik, 2005; Jaouad *et al.*, 2006; Durrington *et al.*, 2001). PON1 hydrolyze organophosphate compounds are widely used as insecticides and nerve gases. Paraoxon is the active metabolite of parathion, an OP pesticide that is widely used in agriculture. Paraoxon undergoes

deactivation through hydrolysis catalyzed by PON1. This enzyme is named from the most commonly used *in vitro* substrate, paraoxon. Therefore, PON1 plays 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. In addition, PON1 is also involved in lipid metabolism, since this enzyme probably hydrolyzes multiple oxygenated forms of polyunsaturated fatty acids of Low Density Lipoproteins (LDLs) associated with phospholipids. For this reason, PON1 can be defined as an antioxidant enzyme (Deakin and James, 2004; Ekinici and Beydemir, 2009). PON1 is widely spread in mammals, such as rat, rabbit and mouse as well as humans. PON1 activity has been found in a variety of mammalian tissues with liver and serum having the highest levels and source of serum PON1 is believed to be primarily the liver (Aldridge, 1953; La Du, 1992). It has been demonstrated that serum PON1 activity was reduced in early postpartum dairy cows (Turk *et al.*, 2004). Many chemicals, especially pesticides, at relatively low dosages affect the metabolism of organisms by altering the activities of enzymes. Different sheep breeds used in this study were Karacabey Merino, Kivircik, Tahirova, Akkaraman and Daglic. The main native sheep breed of Thrace and Marmara regions of Turkey is Kivircik (Kaymakci, 2006). The German Mutton Merino was brought into Turkey in the 1930s to increase live weight and fleece quality of indigenous sheep breeds (Koyuncu and Uzun, 2009). The Karacabey Merino was obtained by crossbreeding the German Mutton Merino with indigenous Kivircik sheep at the Karacabey State Farm (Yalcin, 1986). Akkaraman is a fat-tailed, indigenous breed constituting 45.8% of the sheep population in Turkey (Sahin *et al.*, 2008). Most lamb meat production is from this breed (Akcipinar, 2000). Daglic is grown on the mountainous terrain of the region Ege and Marmara. Daglic body is white and the head and legs are black or brown. The tail is one piece and oval and its shape is like a heart. The spring wool is mixed type, coarse, thin bright. The most preferred wool at weaving carpet is Daglic.

The aim of this study was to determine for the first time, the serum PON1 enzyme activity of five different sheep breeds namely Karacabey Merino, Kivircik, Tahirova, Akkaraman and Daglic. In the experiments, for each breed thirty sheep were used. Kinetic properties of the PON1 enzyme were determined using Lineweaver-Burk graphs. No study has yet been reported on sheep serum paraoxonase.

In this present study, serum paraoxonase activity of Karacabey Merino, Kivircik, Tahirova, Akkaraman and Daglic breeds was first time experimentally investigated.

MATERIALS AND METHODS

The materials used include paraoxon and protein assay reagents were obtained from Sigma Chem. Co and Merck. All other chemicals used were of reagent grade. The blood samples were collected from five different sheep breeds in Balikesir, Turkey.

Collection of blood samples: In the experiments, for each breed thirty sheep were used. The blood samples were collected from each sheep breed within dry tubes. For preparation of serum, the tubes were centrifuged at 5000 rpm for 10 min and the serum was removed. Serum was used for all enzyme assays.

Paraoxonase 1 enzyme assay: Paraoxonase 1 enzyme activity towards paraoxon was quantified spectrophotometrically by the method described by Gan *et al.* (1991). The reaction was followed for 2 min at 37°C by monitoring the appearance of p-nitrophenol at 412 nm in Biotek automated recording spectrophotometer. About 2 mM of final substrate concentration was used during enzyme assay and all measurements were taken in duplicate and corrected for the non-enzymatic hydrolysis.

Kinetic studies and determination of KM and Vmax values: For the kinetic studies of PON1 enzyme activity different concentration of substrate were added to the reaction medium. PON1 activity was assayed by following the hydration of paraoxon. In order to obtain KM and Vmax values of the enzyme using paraoxon as a substrate was measured at seven different substrate concentrations at pH 8.0 and 37°C. KM and Vmax values were determined by means of Lineweaver-Burk graphs. The seven different substrate concentrations, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 mM were added to reaction medium.

RESULTS AND DISCUSSION

The physiological substrates of PON1 are still unknown but structure-reactivity studies and laboratory evolution experiments indicate that the native activity of PON1 is lactonase (Nguyen *et al.*, 2009; Khersonsky and Tawfik, 2006).

PON1 hydrolyzes a wide range of substrates such as esters, thioesters, phosphotriesters, carbonates, lactones and thiolactones. The highest activities observed thus far are with synthetic substrates such as phenyl acetate and dihydrocoumarin and that have no physiological relevance (Draganov and La Du, 2004; Aharoni *et al.*, 2004). It is therefore unlikely that these are PON1's native substrates. Recently, lactonase (lactone

hydrolysis) as well as lactonizing (lactone formation) activities of PON1 were described, including those with lactones of potential physiological relevance such as products of fatty acid oxidation (Billecke *et al.*, 2000; Teiber *et al.*, 2003). These results imply that PON1 might in fact be a lactonase rather than an aryl-esterase or paraoxonase, as traditionally described.

PON1 is a 355 amino acid glycoprotein which is synthesized in the liver and secreted into the blood where it associates with High-Density Lipoproteins (HDLs). PON1 hydrolyzes a wide range of substrates such as esters, thioesters, phosphotriesters, carbonates, lactones and thiolactones. Interestingly, in addition to its role in lipid metabolism, cardiovascular disease and atherosclerosis, PON1 has been shown to play a role in the metabolism of pharmaceutical drugs (Ekinci and Beydemir, 2009).

PON1 has also been shown to be involved in drug metabolism and is used for drug inactivation. *In vitro* assays show that PON1 and PON3 inhibit lipid oxidation in Low Density Lipoproteins (LDLs). Thus, it may be an important detoxifying enzyme and may play a protective role against the development of various diseases. OP compounds are Acetylcholin-esterase (AChE) inhibiting chemicals used as pesticides. Inhibition of acetylcholin-esterase resulting in an accumulation of the neurotransmitter acetylcholine and the continued stimulation of acetylcholine receptors is the suggested mechanism for toxicity of OPs (Rahimi and Abdollahi, 2007). Metabolism of different OPs can give rise to different urinary metabolites diazinon produces only diethylphosphate and diethylthiophosphate (Cocker *et al.*, 2002). OPs are liquids at room temperature and produce a vapor capable of penetrating the skin, respiratory epithelium and cornea (Rahimi and Abdollahi, 2007). The liquid can be absorbed through intact skin and through the gut after ingestion of contaminated food (Evison *et al.*, 2002).

About >100 different OP are active ingredients in pesticide formulations (Chambers *et al.*, 2001). The majority of the OP pesticides belong to the phosphorothioates with a double-bonded sulfur at phosphorus, requiring metabolic activation to give the ultimate active compounds, the oxons.

This desulfuration is mainly brought about by the hepatic CYP450 superfamily, the activity of which is notoriously variable due to genetic polymorphism, enzyme inducers and inhibitors (Eyer *et al.*, 2007).

Beside antioxidant and antiatherogenic properties, PON1 is also a detoxifier that can hydrolyze toxic OPs (Ekinci and Beydemir, 2009). OP oxons are substrates for the plasma enzyme, Paraoxonase 1 (PON1) and this metabolism results in their detoxification. The importance of PON1 in preventing OP toxicity was emphasized by reports showing that mice lacking PON1 were more

susceptible to chlorpyrifos-oxon and diazoxon than wildtype mice (Povey, 2010). It was demonstrated that injection of purified rabbit PON1 provided protection against paraoxon and much better protection against chlorpyrifos/chlorpyrifos oxon exposures, particularly against chlorpyrifos oxon exposures (Li *et al.*, 1993).

Follow-on experiments with genetically modified mice provided convincing evidence that the absence of PON1 in PON1 null mice rendered them dramatically more sensitive to chlorpyrifos oxon exposure with increased sensitivity to high doses of chlorpyrifos (Shih *et al.*, 1998).

The PON1 null mice also showed dramatically increased sensitivity to diazoxon and increased sensitivity to diazinon at high doses (Li *et al.*, 2000). Different pesticides including OPs have been reported to induce oxidative stress due to generation of free radicals and alteration in antioxidant defence mechanisms (Lopez *et al.*, 2007).

The extensive international use of pesticides (mainly organophosphorus compounds, OPs) results in numerous acute intoxications each year (Hernández *et al.*, 2006). The effects of acute pesticide poisoning are well known for those most currently used (Wesseling *et al.*, 1997).

Evidence continues to accumulate that chronic pesticide exposure is associated with impaired health, including carcinogenesis, neurotoxicity, reproductive and development effects and immunological effects (Hernández *et al.*, 2006). Sheep suffer from a number of external parasites which can drastically affect the health of the animals (Trainor *et al.*, 2002).

A number of different products which differ in their effectiveness against different pests are currently licenced for use for the treatment of sheep (Povey, 2010). Pesticide products may contain >1 harmful substance and furthermore in a given proprietary product, the identity and concentration of both active pesticide and formulant may change from year to year (Povey, 2010). Enzymes catalyze almost all chemical reactions in metabolism of the living organisms.

Many chemicals influence metabolism at low concentrations by decreasing or increasing normal enzyme activity, especially by inhibiting specific enzymes with critical function and they are important drug targets (Ekinci and Beydemir, 2009).

The ability of PON1 to hydrolyze several OPs *in vitro* has long been taken as an indication that it may modulate OP toxicity *in vivo* (Costa *et al.*, 2005). PON1 can bind reversibly to organophosphate substrates which it hydrolyzes (Goswami *et al.*, 2009). PON1 is thus the main means of protection of the nervous system against the neurotoxicity of OPs entering the circulation (La Du, 1992).

Furthermore, the presence of polymorphisms in PON1 which confer different enzyme levels and catalytic efficiency have suggested that certain individuals may be more susceptible to the toxic effect of OP exposure (Costa *et al.*, 2005). Two polymorphisms are present in the PON1 coding sequence: a Gln (Q)/Arg(R) substitution at position 192 and a Leu (L)/Met(M) substitution at position 55 (Costa *et al.*, 2005).

The Q/R polymorphism at position 192 significantly affects the catalytic efficiency of PON1 (Costa *et al.*, 2005). Studies indicated that the PON1R192 allozyme hydrolyzes paraoxon more readily than PON1Q192. A series of studies in rodents has provided important evidence to ascertain the role of PON1 in modulating OP toxicity (Costa *et al.*, 2005). PON1 purified from rabbit serum was injected into rats or mice to increase plasma hydrolytic activity toward OP substrates.

When challenged with an OP (chlorpyrifos oxon was used in most instances), animals that had received exogenous PON1 were more resistant than controls to the acute cholinergic toxicity (Costa *et al.*, 2005). No study has yet been reported on sheep serum Paraoxonase 1 (PON1). The present study was initiated to compare the serum PON1 enzyme activities among different sheep breeds namely Karacabey Merino, Kivircik, Tahirova, Akkaramana and Daglic and to emphasize for meat production improvement which breed(s) should be chosen for livestock farming. PON1 enzyme activity was measured as described previously by the following the rate of formation of p-nitrophenol at 412 nm (Gan *et al.*, 1991). In the experiments, for each breed 30 sheepes were used. All enzyme assays were performed in a Biotek automated recording spectrophotometer. Samples were assayed in duplicate and the averaged value was used for analysis. Michaelis-Menten constants (K_M) and maximum Velocities (V_{max}) were determined using paraoxon as a substrate in various different concentrations at 37°C. For each sheep breed, Lineweaver-Burk graphs were drawn and K_M , V_{max} and V_{max}/K_M values of PON1, for paraoxon substrate were calculated from the plots of $1/V$ vs $1/[S]$ (Fig. 1).

As shown in Table 1 for paraoxon substrate, K_M and V_{max} values of Karacabey Merino were 49×10^{-6} mM and 122 U mL^{-1} and of Kivircik were 5.3×10^{-6} mM and 132 U mL^{-1} and of Tahirova were 22×10^{-6} mM and 110 U mL^{-1} and of Akkaraman were 6.7×10^{-6} mM and 95 U mL^{-1} and of Daglic were 17×10^{-6} mM and 57 U mL^{-1} . V_{max}/K_M ratio is called the catalytic power and is a good parameter for finding the most effective substrate. V_{max}/K_M values (of paraoxon substrate were 2.50×10^6 , 24.9×10^6 , 5.0×10^6 , 14.18×10^6 and 3.35×10^6 for Karacabey Merino, Kivircik, Tahirova, Akkaraman and Daglic sheep breeds, respectively. Results of the present study, for the first

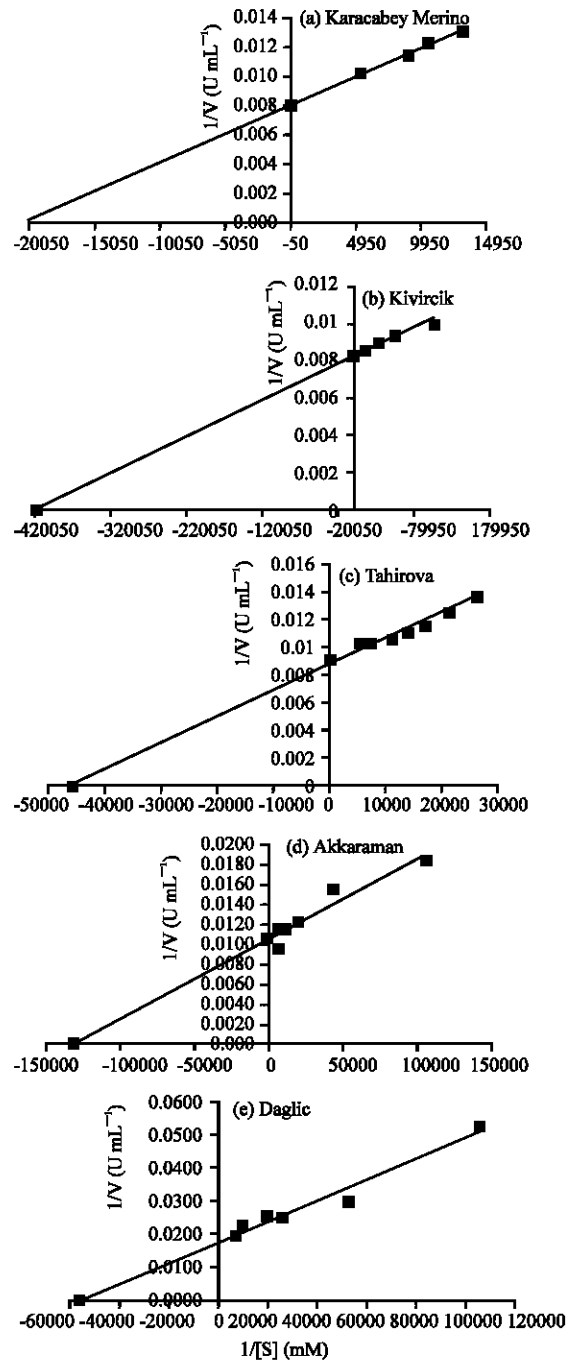


Fig. 1: Lineweaver-Burk graphs for five different sheep breeds such as; a) Karacsbey Merino, b) Kivircik, c) Tahirova, d) Akkaraman and e) Daglic

Table 1: Kinetic values of five different sheep breeds

Sheep breed	K_M (mM)	V_{max} (U mL ⁻¹)	V_{max}/K_M
Karacabey Merino	49×10^{-6}	122	2.50×10^6
Kivircik	5.3×10^{-6}	132	24.9×10^6
Tahirova	22×10^{-6}	110	5.00×10^6
Akkaraman	6.7×10^{-6}	95	14.18×10^6
Daglic	17×10^{-6}	57	3.35×10^6

time showed that serum PON1 activity of five sheep breeds notably changes. According to these results, the values of V_{Max}/K_M showed that Kivircik and Akkaraman breeds have the greatest PON1 activity, on the other hand, Karacabey Merino and Daglic breeds showed the least activity toward paraoxon substrate (Fig. 1 and Table 1). From these results, we can suggest that for sheep livestock farming in Turkey, Kivircik and Akkaraman breeds should be chosen. Since these breeds show higher PON1 activity that means they could show more resistance to OP toxicity. Lack of adequate PON1 activity can effect meat production improvement and could also lead to high mortality of the sheep breed under consideration.

In recent years, increasing attention has been given to biomarkers of susceptibility to OP toxicity. A number of well-established measurements exist to assess exposure and early biological effects (Costa *et al.*, 2005). These include measurements of OP metabolites in the urine of cholinesterase (also known as Butyrylcholinesterase: BuChE) activity in plasma, of Acetyl Cholinesterase (AChE) activity in red blood cells and of NTE in lymphocytes (Maroni *et al.*, 2000).

Because both plasma cholinesterase and erythrocyte acetyl cholinesterase activity are inhibited by Ops. As mentioned before, the importance of PON1 in preventing OP toxicity was highlighted by reports showing that mice lacking PON1 were more susceptible to chlorpyrifos-oxon and diazoxon than wildtype mice (Povey, 2010). Hence in this study, we also claim that measurement of sheep serum blood PON1 activity could be a biomarker of exposure to Ops.

CONCLUSION

In the study, it is founded that PON1 activity may become a sensitive biomarker that can be used together with Acetylcholinesterase (AChE) for the assessment of long-term health risks of sheeps exposed to pesticides.

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