Carbonic Anyhdrase Enzyme From The Siirt Mohair Goat Liver : Purification, Characterization and Assessment of Enzyme Kinetics Against Metal Toxicity

Karbonik Anhidraz Enziminin Siirt Tiftik Keçisi Karaciğerinden Saflaştırılması, Karakterizasyonu ve Metal Toksisitesine Karşı Enzim Kinetiğinin Değerlendirilmesi

Research Article

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ABSTRACT

Because of their physiological and clinical roles, carbonic anhydrases (CAs) are the most studied enzymes. In earlier studies; CA enzymes have been purified and characterized from the tissues and erythrocytes of many organisms such as; dog, swine, sheep, chicken, bee, fish, bovine, bacteria and human. In this study, the CA enzyme has purified from Siirt Mohair Goat liver tissue with 1930.84 EU.mg^{-1} of specific activity, 57.28% of purification yield and 80.55 of purification folds. The purity of the purified enzyme has confirmed by SDS-PAGE. As the characterization of CA enzyme's in Siirt Mohair Goat liver has been done; the optimum ionic strength: 25 mM, the optimum pH: 8.0, the optimum temperature: 40ºC and the stable pH: 7.0 has been determined. Inhibitory effects of some metal ions have been examined on the purified CA enzyme. IC_{50} values of inhibiting metal ions were found as 2.24, 2.76, 2.36, 3.20, 2.55, 2.25, 3.28, 2.13, 3.10, 1.75, 2.16 and 3.50 mM for Al^{3+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Ba^{2+}, Zn^{2+}, B^{3+}, Fe^{3+}, Se^{2+}, Ag^{+} and Co^{2+} respectively. As a result, CA enzyme was first purified from the Siirt Mohair Goat liver and the characteristics of the enzyme were investigated in this study.

Key Words

Affinity, metal ions, inhibition, Siirt mohair goat.

ÖZ

Fizyolojik ve klinik özelliklerinden dolayı karbonik anhidrazlar (CAs), en çok incelenen enzimlerdir. Daha önceki çalışmalarda CA enzimleri köpek, domuz, koyun, tavuk, arı, balık, sığır, bakteri ve insan gibi birçok canlının dokularından ve eritrositlerinden saflaştırılmış ve karakterize edilmiştir. Bu çalışmada da CA enzimi Siirt Tiftik Keçisi karaciğer dokusundan 1930.84 EU.mg^{-1} spesifik aktivite ile %57.28 verimle 80.55 kat saflaştırıldı. Saflaştırılan enzimin saflığı SDS-PAGE ile doğrulandı. Siirt Tiftik Keçisi karaciğerinden CA enziminin karakterizasyonu yapıldığında; optimum iyonik şiddet: 25 mM, optimum pH: 8.0, optimum sıcaklık: 40ºC ve stabil pH: 7.0 olarak belirlendi. Saflaştırılmış CA enzimi üzerine bazı metal iyonlarının inhibitör etkileri incelendi. İnhibisyon gösteren metal iyonlarının IC_{50} değerleri Al^{+3}, Ni^{+2}, Cd^{+2}, Cu^{+2}, Pb^{+2}, Ba^{+2}, Zn^{+2}, B^{+3}, Fe^{+3}, Se^{+2}, Ag^{+} and Co^{+2} için sırasıyla 2.24, 2.76, 2.36, 3.20, 2.55, 2.25, 3.28, 2.13, 3.10, 1.75, 2.16 ve 3.50 mM olarak bulundu. Sonuç olarak bu çalışmada, CA enzimi Siirt Tiftik Keçisi karaciğerinden ilk defa saflaştırıldı ve enzimin karakteristik özellikleri incelendi.

Anahtar Kelimeler

Afinite, metal iyonları, inhibisyon, Siirt tiftik keçisi.

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INTRODUCTION

Carbonic anhydrases (CAs, EC 4.2.1.1) constitute a family of metalloenzymes that are ubiquitously distributed in nature and are responsible for catalyzing the reversible interconversion between carbon dioxide and bicarbonate. They are involved in a variety of biosynthetic reactions like gluconeogenesis, lipogenesis and ureagenesis as well as in numerous physiological processes including respiration, secretion of electrolytes and pH regulation. In this regard, their key function is to maintain acid base balance, which is of particular importance for the normal growth of cell [1]. The physiological function of the CA isozymes is to facilitate the interconversion of CO_2 and HCO_3^-; therefore, they play key roles in diverse processes, such as physiological pH control and gas balance, calcification and photosynthesis. Also, CA plays an important role in ion transport and pH regulation in eye, kidney, central nervous system and inner ear [2]. Heavy metals are among the most toxic and unavoidable contaminants of earth. Heavy metals are an important problem in environmental toxicology. Most of the heavy metals are toxic to humans, animals and plants. Metals are natural trace components of the aquatic environment, but their levels have increased due to industrial, agricultural and mining activities [3,4]. This situation may be hazardous for living systems, especially aquatic organisms, including specific enzymes. It is well known that enzymes catalyse almost all chemical reactions in the metabolism of the living systems. These chemical substances including pollutants, pesticides, drugs and metal ions influence metabolism at low concentrations by decreasing or increasing enzyme activities [5]. Specifically, some enzymes including carbonic anhydrase are considered drug- and chemical-targeted enzymes. For example, CA is expressed in almost all the tissues of living things. Because of these, the enzyme, which has great importance in the balance of pH and respiration in various tissues, is a known target enzyme for different substances [5-7]. The toxicological effects of heavy metals are usually enzyme inhibition and denaturation. Generally, the mechanism underlying metal inhibition of the enzyme is based on heavy metal binding to the protein [8]. Heavy metals can alter enzymatic activities by binding to functional groups, such as sulfhydryl, carboxyl and imidazole, or by displacing the metal associated with the enzyme [9]. Besides, CA inhibitors are some of the principal drugs used in the management of glaucoma. Mohair goat is one of the oldest living animal genes. There are traces of this type of goat at old records in BC 11th, 12th and even 14th centuries. Mohair goat is a small, slim and elegant race. Mohair goat has a soft body, covered with bright and curly mohair. Mohair goat was only grown in Anatolia until 1838 then it started to be grown in several countries, mainly in South Africa. Today, mohair goat and mohair producing countries are South Africa, the United States and Turkey. Except these countries, mohair goats are grown in Argentina, Lesotha, Canada, New Zealand, Russia and in Brazil. But numbers are declining rapidly due to economic reasons [10]. The aim of this study was to determine the effects of certain metals on CA enzyme that is characterized and purified from Siirt Mohair Goat liver tissue.

MATERIALS and METHODS

Chemicals

4-nitrophenylacetate, sepharose 4B, protein assay reagents were obtained from Sigma Aldrich Co. All other chemicals were of analytical grade and obtained from Merck.

Purification of Carbonic Anhydrase from Siirt Mohair Goat Liver Tissue by Affinity Chromatography

The 30 g tissue sample was centrifuged at 10.000 rpm for 30 min and the plasma and precipitate were removed. The pH of the homogenate obtained from Siirt Mohair Goat liver tissue was adjusted to 8.7. Homogenate was applied to the column and washed with a solution of 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). Thus, the enzyme CA was attached to the column and other impurities were removed. Then, 0.1 M $NaCH_3COO.3H_2O$/0.5 M $NaClO_4$ (pH 5.6) buffer was applied to the column to eluate CA enzyme. Eluates were taken into the tubes with a fraction collector and absorbance of the tubes was measured at 280 nm for qualitative protein determination. All procedures were performed at 4°C.

Esterase Activity Assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (CHEBIOS UV–vis) according to the method described by Verpoorte et al. [11].

Protein Determination

The purification steps of the protein was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard [12].

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzymes. It was carried out in 10% and 3% acrylamide for the running and the stacking gel, respectively, containing 0.1% SDS according to Laemmli procedure. A 20 mg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coommassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye [13].

Optimum pH Determination

In order to determine the optimum pH, K-phosphate and Tris–HCl buffers were used in the pH range of 5.0-7.5 and 7.0-9.0, respectively.

Optimum Temperature Determination

For determination of the optimum temperature, enzyme activity was assayed at different temperatures in the range from 10°C to 80°C. The desired temperature was provided by using a Grant bath.

Optimum Ionic Strength Determination

For determination of optimum ionic strength, enzyme activity was determined using different concentrations of Tris-HCl buffer, pH: 8.0, in the range from 10 mM to 1000 mM.

Stable pH Determination

For this aim, equal volumes of the buffers Tris-HCl at pH of 7.0, 7.5, 8.0, 8.5 and 9.0, and purified enzyme were mixed and kept in a refrigerator (+4°C). The enzyme activity was assayed at 12 hour intervals.

***In Vitro* Effects of Metal Ions**

In order to determine the effects of the metal ions on Siirt Mohair Goat liver CA, different concentrations of metal ions were added to the reaction medium. The inhibitory effects of Al^{3+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Ba^{2+}, Zn^{2+}, B^{3+}, Fe^{3+}, Se^{2+}, Ag^{+} and Co^{2+} were examined. All compounds were tested in triplicate at each concentration used. Different inhibitor concentrations were used. Control cuvette activity in the absence of inhibitor was considered as 100%. For each inhibitor, an activity (%)–[inhibitor] graph was drawn [14].

RESULTS

Carbonic anhydrase from Siirt Mohair Goat liver was purified up to 80.55-fold with a recovery ratio of 57.28% compared to homogenate (Table 1).

After the sample had completely passed through, the column was washed with 25 mM Tris-HCl/22 mM Na_2SO_4 buffer whose pH was (8.7). During washing, absorbencies of fractions were spectrophotometerically measured at 280 nm and 348 nm. These values of the absorbance showed that some proteins, bound to the affinity material, have been removed from the column by the washing solutions. Then, the enzyme was eluted with 0.1 M

Table 1. Summary of purification procedure of CA enzyme from Siirt Mohair Goat liver.

Purification Steps	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity	Specific activity (EU/mg)	Yield (%)	Purification fold
Homogenate	643	20	26.84	536.8	12866	23.97	100.0	1.0
Sepharose-4B-L tyrosine-sulfanilamide affinity chromatography	670	11	0.35	3.82	7370	1930.84	57.28	80.55

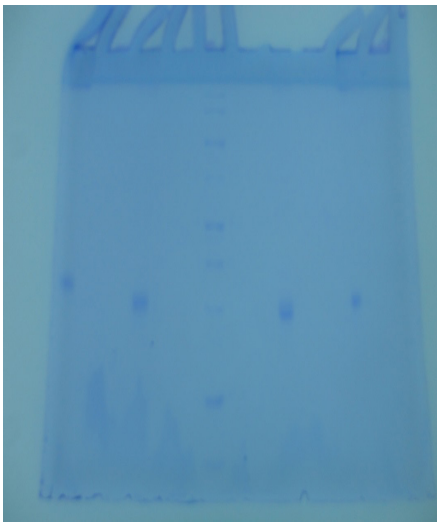

Figure 1.: SDS-PAGE analysis of purified CA enzyme from Siirt Mohair Goat liver. Lane (1) Siirt Mohair Goat liver CA enzyme. Lane (2) standard proteins (250 kDa), (150 kDa), (100 kDa), (70 kDa), (50 kDa), (30 kDa), (15 kDa).

$NaCH_3COO.3H_2O$/0.5 M $NaClO_4$ (pH 5.6). At the end of the last step, a highly purified enzyme was obtained exhibiting a single band on SDS-PAGE (Figure 1).

Besides, some kinetic properties of the enzyme was investigated. The optimum pH, optimum temperature, optimum ionic strength and stable pH were determined to be 8.0, 40°C, 25 mM and 7.0 for the enzyme, respectively (Table 2).

The stable pH profile of the enzyme was determined at five different pH's in 25 mM Tris-HCl. The enzyme was able to protect 76.2 % of maximum activity at the end of 72 hours in 25 mM Tris-HCl buffer (pH: 7.0). These results are similar to CA obtained from Agrı Balık Lake Trout Gill [15]. The enzyme was seen to exhibit the highest activity at 40°C in a study of temperatures between 10°C and 80°C. The optimum ionic strength of the enzyme was estimated to be 25 mM Tris-HCl buffer. However, about 85% of the maximum activity was present in the broad range from 10 mM to 1000 mM. In addition to establishment of molecular weight of the enzyme, R_f values were also calculated for the enzyme and standard proteins, and R_f–LogMW graph was obtained according to Laemmli' method [13]. The molecular weight of the enzyme was determined to be 29.5 kDa. K_M and V_{max} values were calculated for NPA by Lineweaver–Burk graph (Figure 2).

Table 2. Kinetic properties of CA enzyme from Siirt Mohair Goat liver.

Substrate	p-Nitrophenyl acetate
Optimum Ionic Strength (mM)	25.0
Optimum pH	8.0
Optimum Temperature (°C)	40.0
Stable pH	7.0
K_M (mM)	0.79
V_{max} (μmol.min^{-1})	0.74

K_M constants were calculated as 0.794 mM, V_{max} values as 0.736 mol.min^{-1} for NPA (Table 2). In addition to purification and characterization of the enzyme, Al^{3+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Ba^{2+}, Zn^{2+}, B^{3+}, Fe^{3+}, Se^{2+}, Ag^{+} and Co^{2+} metal ions were chosen to investigate their inhibitory effects on Siirt Mohair Goat liver CA. IC_{50} parameters of these metals were determined. Metal ions inhibited the enzyme activity at low concentrations (Table 3).

In vitro inhibition rank order was determined as $Se^{2+} > B^{3+} > Ag^{+} > Al^{3+}$ $Ba^{2+} > Cd^{2+} > Pb^{2+} > Ni^{2+} > Fe^{3+} > Cu^{2+} > Zn^{2+} > Co^{2+}$.

DISCUSSION

CA has been purified from many different tissues up to now; including human erythrocytes [16], sheep liver [17], fish liver [5,18], fish gills [15,19], rainbow trout liver and kidney [7,20], bees [21]. Although, CA and the inhibitory effects of many metals on CA have been studied in most tissues, no study has been done on Siirt Mohair Goat liver, yet. For this reason, Siirt Mohair Goat liver CA

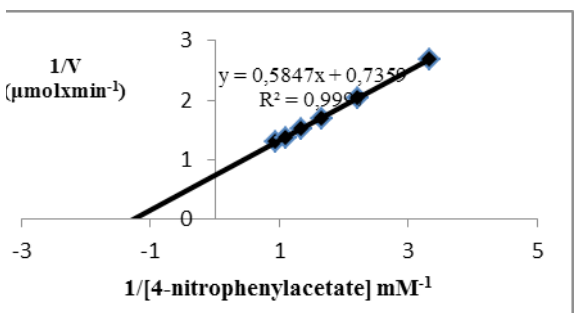

Figure 2. Lineweaver–Burk graph with five different 4-nitrophenylacetate concentrations.

Table 3. Inhibition values of metal ions on CA enzyme from Siirt Mohair Goat liver.

Inhibitors	IC_{50}	Inhibitors	IC_{50}
Al^{3+}	2.24mM	Se^{2+}	1.75 mM
Ni^{2+}	2.76 mM	Ba^{2+}	2.25 mM
Zn^{2+}	3.28 mM	B^{3+}	2.13 mM
Cu^{2+}	3.20 mM	Fe^{3+}	3.1 mM
Cd^{2+}	2.36 mM	Ag^{1+}	2.16 mM
Pb^{2+}	2.55 mM	Co^{2+}	3.5 mM

enzyme was isolated and characterized for the first time in this study. Purification procedure was carried out by affinity chromatography on Sepharose 4B tyrosine-sulfanilamide. We achieved to purify the enzyme in a single step. The molecular weight of the enzyme was determined to be 29.5 kDa. Similar results have been observed for the enzyme from different sources. For example, sheep kidney CA is 28.9 kDa [18], the teleost fish D. labrax liver CA is also of 29 kDa [5], human erythrocyte CA is 29 kDa [16], bovine erythrocyte CA is 29 kDa [6] and rainbow trout liver CA is 31 kDa [20].

It is clear that Se^{2+} and B^{3+} are the most potent inhibitors for Siirt Mohair Goat liver CA enzyme. CA has histidines exposed on the surface. The mechanism of toxicological effects of the metals is probably due to the interactions between the histidines and the metals or the interaction of the metals with other aminoacids around the active site. The interactions between specific enzyme systems and heavy metals ions have been extensively studied in recent years. Demirdag et al. [17] investigated the inhibition effects of some metal concentrations (Cu^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+} and Ni^{2+}) on the activity of CA from sheep liver. *In vitro* inhibition rank order was determined as Cu^{2+}> Zn^{2+}> Cd^{2+}> Co^{2+}>Ni^{2+}. Söyüt and Beydemir [7] investigated the *in vitro* effects of Co^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+} and Ag^{+} on chemical-targeted CA enzyme from rainbow trout kidney. *In vitro* inhibition rank order was determined as Co^{2+}>Zn^{2+}>Cu^{2+}>Cd^{2+}>Ag^{+}. Ceyhun et al. [5] investigated the inhibition effects of some metal concentrations (Al^{3+}, Cu^{2+}, Pb^{2+}, Co^{3+}, Ag^{+}, Zn^{2+} and Hg^{2+}) on the activity of CA from the teleost fish *Dicentrarchus labrax* liver. *In vitro* inhibition rank order was determined as Al^{3+}>Cu^{2+}>Pb^{2+}> Co^{3+}> Ag^{+}> Zn^{2+}> Hg^{2+}. In our this study *in vitro* inhibition rank order was determined also as Se^{2+} > B^{3+} > Ag^{+} > Al^{3+} Ba^{2+} > Cd^{2+} > Pb^{2+} > Ni^{2+} > Fe^{3+} > Cu^{2+} > Zn^{2+} > Co^{2+}. The results obtained in our work are similar to data reported by many researchers [3,5,7,17-20,22]. In recent years, people have become concerned about the water, soil and air pollution by various heavy metals. Because, some heavy metals (nickel, silver and cadmium) that enter the food chain are not discharged from living things. Therefore these heavy metals are accumulated in living things bodies. Then, they can be ingested by humans via the consumption of nutrients.

Consequently, we purified carbonic anhydrase from Siirt Mohair Goat liver for the first time, and analyzed characteristic features of the enzyme. Our results are in good agreement with others reported in literature. In addition, inhibitory effects of metals on enzyme activity were reported. Thus, the characteristics of the enzyme and the inhibition of the enzyme against metals have been added to the literature.

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References

1. A. Saeed, S.U. Khan, P.A. Mahesara, P.A. Channar, G. Shabir, J. Iqbal, Substituted (E)-2-(2-benzylidenehydrazinyl)-4-methylthiazole-5-carboxylates as dual inhibitors of 15-lipoxygenase & carbonic anhydrase II: synthesis, biochemical evaluation and docking studies, Biochem. Biophys. Res. Commun., 482 (2017) 176-181.
2. D. Ekinci, H. Cavdar, O. Talaz, M. Senturk, C.T. Supuran, NO-releasing esters show carbonic anhydrase inhibitory action against human isoforms I and II, Bioorg. Med. Chem., 18 (2010) 3559–3563.
3. E.D. Kaya, H.Soyut, S.Beydemir, Carbonic anhydraseactivity from the gilthead sea bream (*Sparus aurata*) liver: the toxicological effects of heavy metals, Environ. Toxicol. Pharmacol., 36 (2013) 514–521.
4. M. Kalay, M.Canlı. Elimination of essential (Cu, Zn) and nonessential (Cd, Pb) metals from tissues of a freshwater fish *Tilapia zillii* following an uptake protocol, Turk. J. Zoology, 24 (2000) 429–436.
5. S.B. Ceyhun, M. Senturk, E. Yerlikaya, O. Erdogan, O.I. Kufrevioglu, D. Ekinci, Purification and characterisation of carbonic anhydrase from the teleost fish *Dicentrarchus labrax* (European sea bass) liver and toxicological effects of metals on enzyme activity, Environ. Toxicol. Pharmacol., 32 (2011) 69-74.

6. R. Demirdag, E. Yerlikaya, E. Aksakal, O.I. Kufrevioglu, D. Ekinci, Influence of pesticides on the pH regulatory enzyme, carbonic anhydrase, from European Seabass liver and bovine erythrocytes, Environ. Toxicol. Pharmacol., 34 (2012) 218–222.
7. H. Soyut, S. Beydemir, The impact of heavy metals on the activity of carbonic anhydrase from Rainbow trout (*Oncorhynchus mykiss*) kidney, Toxicol. Ind. Health, 28 (2012) 296–305.
8. D. Ekinci, S. Beydemir, Risk assessment of pesticides and fungicides for acid-base regulation and salt transport in rainbow trout tissues, Pestic. Biochem. Phys., 97 (2010) 66–70.
9. A.Viarengo, Biochemical effects of trace metals, Mar. Pollut. Bull., 16 (1985) 153–158.
10. G. Kanturk Yigit, Angora goat and Mohair Production in Turkey, Arch. Appl. Sci. Res., 3 (2011) 145-153.
11. A.J. Verpoorte, S. Mehta, J.T. Edsall, Esterase activities of human carbonic anhydrases B and C, J. Biol. Chem., 242 (1967) 4221–4229.
12. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding, Anal. Biochem., 72 (1976) 248.
13. D.K. Laemmli, Cleavage of structural proteins during in assembly of the head of Bacteriophage T4, Nature, 227 (1970), 680-685.
14. H. Lineweaver, D. Burk, The determination of enzyme dissociation constants, J. American Chem. Soc., 56 (1934) 685-686.
15. R. Demirdag, V. Comakli, M. Kuzu, E. Yerlikaya, M. Senturk, Purification and Characterization of Carbonic Anhydrase from Agrı Balık Lake Trout Gill (*Salmo trutta labrax*) and Effects of Sulfonamides on Enzyme Activity, J Biochem Molecular Toxicology, 29 (2015) 123-128.
16. M. Senturk, I. Gulcin, A. Dastan, O.I. Kufrevioglu, C.T. Supuran, Carbonic anhydrase inhibitors. Inhibition of human erythrocyte isozymes I and II with a series of antioxidant phenols, Bioorg. Med. Chem., 17 (2009) 207–211.
17. R. Demirdag, E. Yerlikaya, O.I. Kufrevioglu, Purification of carbonic anhydrase-II from sheep liver and inhibitory effects of some heavy metals on enzyme activity, J. Enzyme Inhib. Med. Chem., 27 (2012) 795-799.
18. R. Demirdag, E. Yerlikaya, M. Senturk, O.I. Kufrevioglu, C.T. Supuran, Heavy metal ion inhibition studies of human, sheep and fish α-carbonic anhydrases, J. Enzyme Inhib. Med. Chem., 28 (2013) 278–282.
19. E.D. Kaya, H. Soyut, S. Beydemir, The toxicological impacts of some heavy metals on carbonic anhydrase from gilthead sea bream (*Sparus aurata*) gills, Environ. Toxicol. Pharmacol, 39 (2015) 825–832.
20. H. Soyut, S. Beydemir, Purification and some kinetic properties of carbonic anhydrase from rainbow trout (*Oncorhynchus mykiss*) liver and metal inhibition, Protein Peptide Lett, 15 (2008) 528–535.
21. E. Soydan, A. Güler, S. Bıyık, M. Senturk, C.T. Supuran, D. Ekinci, Carbonic anhydrase from Apis mellifera: purification and inhibition by pesticides, J. Enzyme Inhib. Med. Chem., 32 (2017) 47-50.
22. R. Demirdag, E. Yerlikaya, O.I. Kufrevioglu, C. Gundogdu, Purification of glutathione S-transferase isoenzymes from tumour and nontumour human stomach and inhibitory effects of some heavy metals on enzymes activities, J. Enzyme Inhib. Med. Chem., 28 (2013) 911-915.