Muş Alparslan Üniversitesi Fen Bilimleri Dergisi
Muş Alparslan University Journal of Science
ISSN:2147-7930

Cilt/*Volume***:2 Sayı/** *Issue***:2 Aralık/***December***: 2014**

PURIFICATION AND CHARACTERIZATION OF GLUTATHIONE S- TRANSFERASE FROM LAUREL FRUIT *(Laurocerasus officinalis Roem.)* AND INHIBITION THE EFFECTS OF SOME PESTICIDES ON ENZYME ACTIVITY

KARAYEMİŞ MEYVESİNDEN *(Laurocerasus officinalis Roem.)* GLUTATYON S-TRANSFERAZ ENZİMİNİN SAFLAŞTIRILMASI VE KARAKTERİZASYONU VE BAZIPESTİSİTLERİN ENZİM AKTİVİTESİNE İNHİBİSYON ETKİLERİ

Fikret TÜRKAN[1]*, Neslihan BALCI[2], Halis ŞAKİROĞLU[2]

[1]*Igdır University, Health Services Vocational School, ,76100 Igdır,fikret.turkan@gmail.com*
[2]*Atatürk University, Department of Biochemıstry, Faculty of Science, 25240 Erzurum, Turkey*

ABSTRACT

In this study; the glutathione S-transferase (EC 2.5.1.18) was prufied and characterized from the laurel fruit (Laurocerasus officinalis Roem.). Purification was performed in one step by affinity chromatography. SDS-polyacrylamide gel electrophoresis was applied for control of the glutathione S-transferase. In kinetic studies on the enzyme; optimum pH, optimum temperature, optimum ionic strength, K_M and V_{max} for reduced glutathione (GSH) and 1- chloro 2,4- dinitrobenzene (CDNB) studies were carried out. According to the results, K_M and V_{max} values for GSH 0.194 mM and 0.038 EU/ml, K_M and V_{max} values for CDNB 0.353 mM and 0.099 EU/ml. The effect of pesticides were researched on enzyme activity. Of these pesticides; dichlorvos, cypermethrin, imidacloprid, fenoxaprop-p-ethyl, glyphosate isopropilamine salt's to exhibit inhibitory properties; haloxyfop-p-methyl, lambda-cyhalothrin and 2,4-dichlorophenoxyasetic acid dimethylamine salt's were found to act as the activator.

Keywords: *Glutathione S-Transferase, Glutathione, Inhibition, Purification, Laurel fruit*

ÖZET

Bu çalışmada karayemiş (Laurucerasus officinalis Roem.) meyvesinden Glutatyon S- Transferaz (EC 2.5.1.18) enzimi saflaştırıldı ve karakterize edildi. Saflaştırma işlemi tek basamakta afinite kromatografisiyle yapıldı. GST enziminin saflığını kontrol etmek için SDS-PAGE elektroforezi uygulandı. Enzim ile ilgili kinetik çalışmalar olarak optimum pH, optimum sıcaklık, optimum iyonik şiddet ile indirgenmiş glutatyon (GSH) ve 1- kloro 2,4- dinitrobenzen (CDNB) için K_M ve V_{max} çalışmaları yapıldı. Çalışma sonuçlarına göre GSH için K_M ve V_{max} değerleri 0.194 mM ve 0.038 EU/ml, CDNB için K_M ve V_{max} değerleri 0.353 mM ve 0.099 EU/ml olarak bulundu. Enzim aktivitesi üzerine bazı pestisitlerin etkisi araştırıldı. Bu pestisitlerden; diklorvos, cypermethrin, imidacloprid, fenoxaprop-p-ethyl, glyphosate isopropilamin tuzunun inhibitör özelliği gösterdiği; haloxyfop-p-methyl, lambda- cyhalothrin ve 2,4- dichlorophenoxyasetic asit dimethylamine tuzunun ise aktivatör olarak davrandığı tespit edildi.

Anahtar kelimeler: *Glutatyon S-Transferaz, Glutatyon, İnhibisyon, Saflaştırma, Karayemiş*

***Corresponding author:** Fikret TÜRKAN, Igdır University, Health Services Vocational School, Karaağaç Kampüsü,76000 Igdır,Turkey E-mail: fikret.turkan@gmail.com

1. INTRODUCTION

Laurocerasus officinalis Roem. cherry laurel, locally named "karayemiş", is a summer fruit highly characteristic of the Black Sea region [1]. It is known for its unique taste and ethnopharmacological uses including its diuretic and antidiabetic properties and for treatment of stomach ulcers, digestive system problems, bronchitis, eczemas, and hemorrhoids [2,3]. *L. officinalis*, also known as taflan or wild cherry. It is produced in the eastern Black Sea region of Turkey, some of the Balkans, Northern Ireland, western Europe, southern and western Caucasia, Iran, eastern Marmara, some Mediterranean countries.

The cherry laurel tree is an evergreen plant of 6 m height with ovoid dark purple to blackish fruits 8-20 mm in diameter [1]. Plantings of *L. officinalis* contribute to reduction of the risk of desertification due to its evergreen nature, and its leaves present an alternative use in landscape architecture. The fruit is consumed directly both fresh and dried as well as in the form of jam, pulp, marmalade, and drinks [4]. *L. officinalis*, as many plants do, can synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives.

The phenolic content and composition of fruits and vegetables depend on genetic and environmental factors as well as postharvest processing conditions [5]. Plant phenolics are the largest class of plant secondary metabolites, which, in many cases, serve in plant defense mechanisms to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage and damage by microorganisms, insects, and herbivores [6]. Plant phenolics are found mainly in vacuoles. The antioxidant activity of phenolics is related to a number of different mechanisms such as free radical scavenging, hydrogen donation, singlet oxygen quenching, metal ion chelation, and acting as a substrate for radicals such as superoxide and hydroxide [5]. A direct relationship has been found between the phenolic content and antioxidant capacity of many fruits and vegetables. Plant antioxidants have also been shown to present synergy [7]. Ascorbic acid, the most well-known antioxidant, is an important molecule in plant tissues and protects plants against oxidative damage resulting from the oxidant metabolites of photosynthesis and aerobis processes [8]. Oxidative stress is well-known to cause many disases [9] scientists have become more interested in natural sources to fight it, looking for active components of plants in this respect in the recent years [10,11].

Glutathione S-transferases (GSTs; EC 2.5.1.18), are a large family of multifunctional enzymes found ubiquitously in aerobic organisms [12] . They play important roles in phase II detoxification of several chemical insecticide classes, i.e., pyrethroids [13]. Organophosphates [14], and chlorinated hydrocarbons such as DDT [15]. In insects, high levels of GSTs activity are associated with the expression of metabolic resistance to insecticides [16,17]. Most GST described to date are dimers composed of 22–30 kDa subunits, each subunit has a glutathione (GSH) binding site (G-site) and an adjacent electrophilic substrate binding site (H-site) [18, 19]. These enzymes are mainly found in cytosol of organisms and may be divided into several classes such as phi (F), tau (U), theta (T), zeta (Z), lambda (L) and dehydroascorbate reductase (DHAR), among which phi and tau classes are specific to plants [20]. Based on protein sequence alignments, 40 of the genes assigned to the phylogenetic class tau, 16 to phi, 3 to zeta, 2 to theta in laurel fruit. In addition, these GST distribute in different organs and tissues in rice, and the expression of specific GST vary differently during plant development [21].

Laurel fruit, is an important crop sustaining human society, providing food for the majority the global population. However, contamination of orchards by pesticides such as diklorvos, cypermethrin, imidacloprid, fenoxaprop-p-ethyl, glyphosate isopropilamine salt's due to industrial and agricultural activities has become a global environmental problem, posing risk to

human health. Therefore, in this study, we aimed to purify and identify GST isozymes in laurel fruit upon pesticides exposure using chromatography, electrophoresis and mass spectrometry.

2. MATERIALS AND METHODS

2.1. Materials

Glutathione-agarose was purchased from Sigma-Aldrich. Sephadex G-100, GSH, CDNB, the protein assay reagent and the chemicals for electrophoresis were purchased from Sigma Chem. Com. All other chemicals used were analytical grade and purchased from either Sigma or Fluka. Ripe fruits of *L. officinalis* were collected from Erbaa, Tokat, Turkey, after full ripening in September 2013. They were kept in cool bags for transport to the laboratory. The fruits were stored as packed in freezer bags at -20 °C until tested.

2.2. Preparation of the Homogenates

The laurel fruit was powdered in a mortar filled with liquid nitrogen, and the powder was suspended in 0,2 mol/L Tris-HCl buffer (pH 7.8, containing 5 mmol/L DTT, 1 mmol/L EDTA and 1 mmol/L PMSF). The crude extract was filtered through four layers of cheese cloth and then centrifuged for 30 min (4 °C, 20.000xg) and then the precipitate was removed.

2.3. Glutathione-agarose Affinity Chromatography

Purification of the GST from the laurel fruit was performed with Glutathione-Agarose affinity gel [22]. For this aim, two gram of Glutathione-Agarose as a lyoplihized powder stabilized with lactose was used for approximately 14 ml column size. The lyoplihized powder was incubated a night in deionized water and swelled. After swelling, the gel was washed thoroughly with 10 ml volumes of deionized water to remove the lactose present in the lyoplihized product and packed in a column. After precipitation of the gel, it was equilibrated 10 mM phosphate buffer including 150 mM NaCl, pH:7.4, (equilibration buffer) by means of a peristaltic pump [23,24].

The flow rates for washing and equilibration were adjusted 20 ml/h. The previously prepared homogenate was loaded onto the Glutathione-Agorose column and washed with equilibration buffer until the final absorbance difference became 0.05 at 280 nm. The enzyme was eluted successively with a gradient of 0 to 10 mM GSH in 50 mM Tris-HCl buffer (pH: 9.5 containing 30 mmol/L GSH, 5 mmol/L DTT and 1 mmol/L EDTA). Active fractions were collected and dialyzed with equilibration buffer. All of the procedures were performed at 4 °C [25-29]

2.4. Activity Determination

GST activity with different substrates was determined as described by Habig et all. [27].

2.5. Protein Determination

Protein content of the homogenate was determined according to the method of Bradford (1976) [28] with bovine serum albümin as a standard. The color of the reaction was measured spectrophotometrically at 595 nm wavelengeth at 25 °C.

2.6. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To determine the enzyme's purity, SDS-PAGE was performed according to Laemmli's method. The acrylamide concentration of the stacking and separating gels were 3% and 15%, respectively, and 1% SDS was also added to the gel solution. The gel was stained for 2 h in 0.1% Coommasie Brillant Blue R-250 containing 50% methanol, 10% acetic acid and 40% distilled water, then destained with many changes of the same solvent without dye. Cleared protein bands were photographed

(Fig. 1).

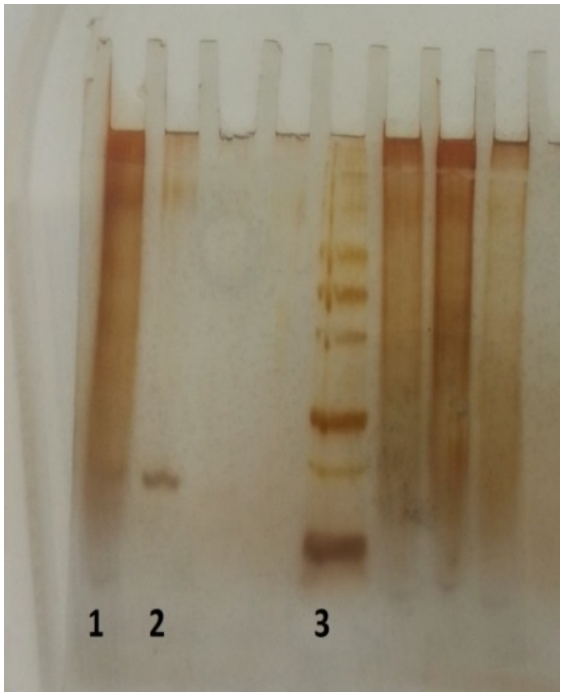


Figure 1. SDS- PAGE photograph: (1: homogenate, 2: purified enzyme from glutathione-agarose matrix, 3:standart proteins.

2.7. Determination of Optimum pH

To determine optimal pH, the enzyme activity was measured in 0.1 M Tris- HCl and potassium phosphate buffers over the pH ranges 7-9 and 5-8 respectively (Fig.2).

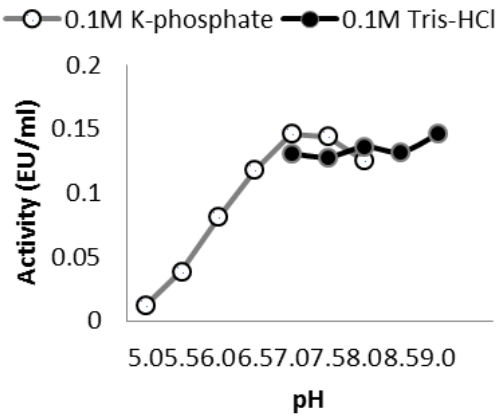


Figure 2- Effect of pH on activity of laurel fruit GST. The buffer used were 0.1 M K-phosphate buffer (pH 5-8) and Tris-HCl buffer (pH 7-9)

2.8. Determination of Optimum Ionic Strength

Effect of ionic strength on the enzyme was determined in a range from 0.0625 to 1.5 M K-phosphate (pH: 7) buffer concentrations at the assay conditions.(Fig.3).

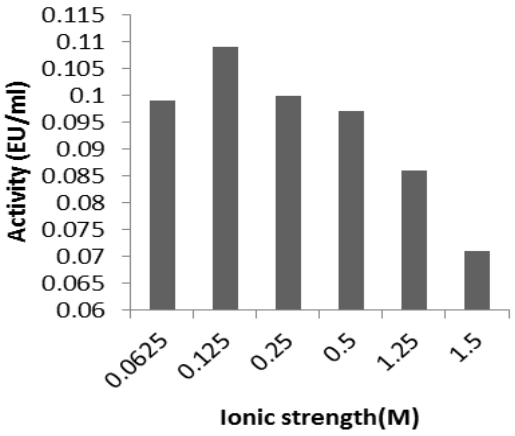


Figure 3. Effect of ionic strenght on activity of laurel fruit GST. The activity was assayed in different concentrations between 0.0625 and 1.5 M K-Phosphate buffer at room temperature**.**

2.9. Determination of Optimum Temperature

To determine optimum temperature of laurel fruit GST, enzyme activity was assayed in 0.1 M K- phosphate buffer (pH: 7) at different temperatures in a range from 0 °C to 75 °C (Fig.4). The desired temperature was provided by using water bath (Nuve; model BM-302).

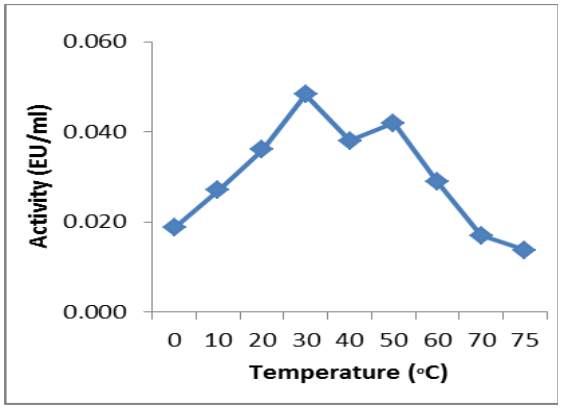


Figure 4. Effect of temperature on activity of laurel fruit GST. The activity was assayed in 0.1 M K-Phosphate buffer (pH 7)

2.10. Determination of Stable pH

To determine pH stability of the enzyme, equal volumes of purified enzyme sample and the buffers (0.1 M K- phosphate at pH of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 0.1 M Tris- HCl at pH of 7.0, 7.5, 8.0, 8.5, 9.0) were mixed and kipt in refrigerator (+4 °C). The enzyme activity was measured 4 days with 12 hours interval.

2.11. Kinetic Studies

The kinetic parameters, V_{max} and K_M for CDNB were determined using a CDNB range from 0.05 mM to 1.6 mM and a fixed GSH concentration of 20 mM (Fig.5a). The apparent V_{max} and K_M values for GSH were also calculated by using a GSH range from 0.05 mM to 1.6 mM and a fixed CDNB concentration of 25 mM (Fig. 5b).

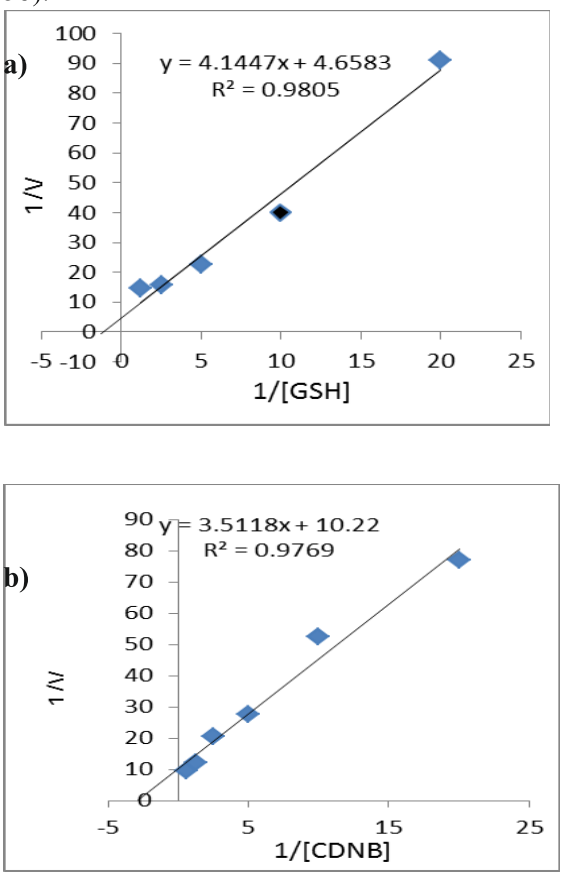
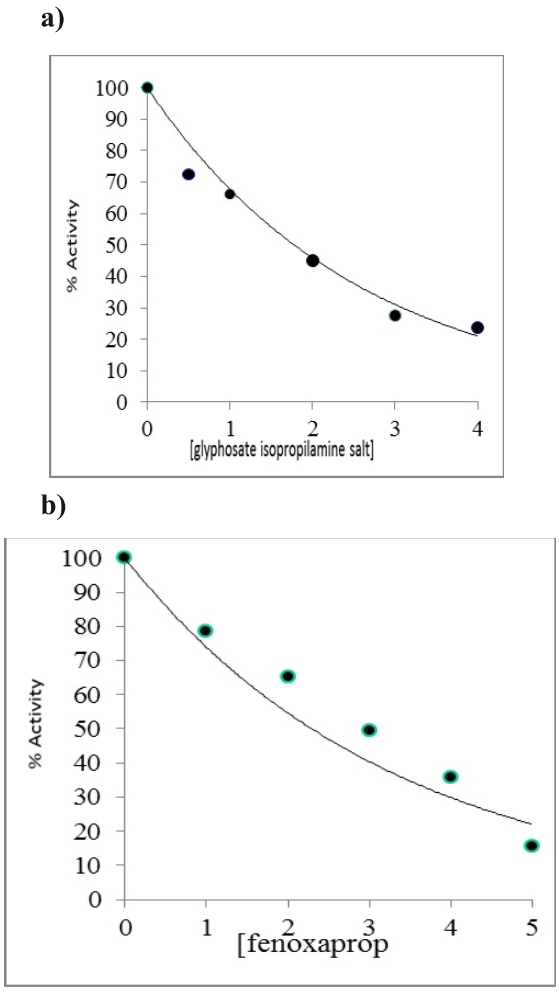


Figure 5. Kinetic analysis of laurel fruit GST activity. The activity was assayed in the ranging from (a,b) 0.025 mM to 3.2 mM CDNB with GSH.

2.12. *In vitro* Inhibition Assay

The *in vitro* inhibition assay was performed using the GSTs assay conditions in the absence or presence of various concentrations of inhibitors. Stock solutions of the inhibitors, including dichlorvos, cypermethrin, imidacloprid, fenoxaprop-p-ethyl, glyphosate isopropilamine salt. The following concentrations of diklorvos (0.5 mM - 3.5 mM), cypermethrin (0.1 mM - 0.6 mM), imidacloprid (0.1 mM – 0.6 mM), fenoxaprop-p-ethyl (1 mM – 5 mM), and glyphosate isopropilamine salt (0.5 mM – 4 mM) were added into the measurement cuvette. Activity of the enzyme was measured, and the absence of pesticides was used as control (100% activity) (Fig.6).



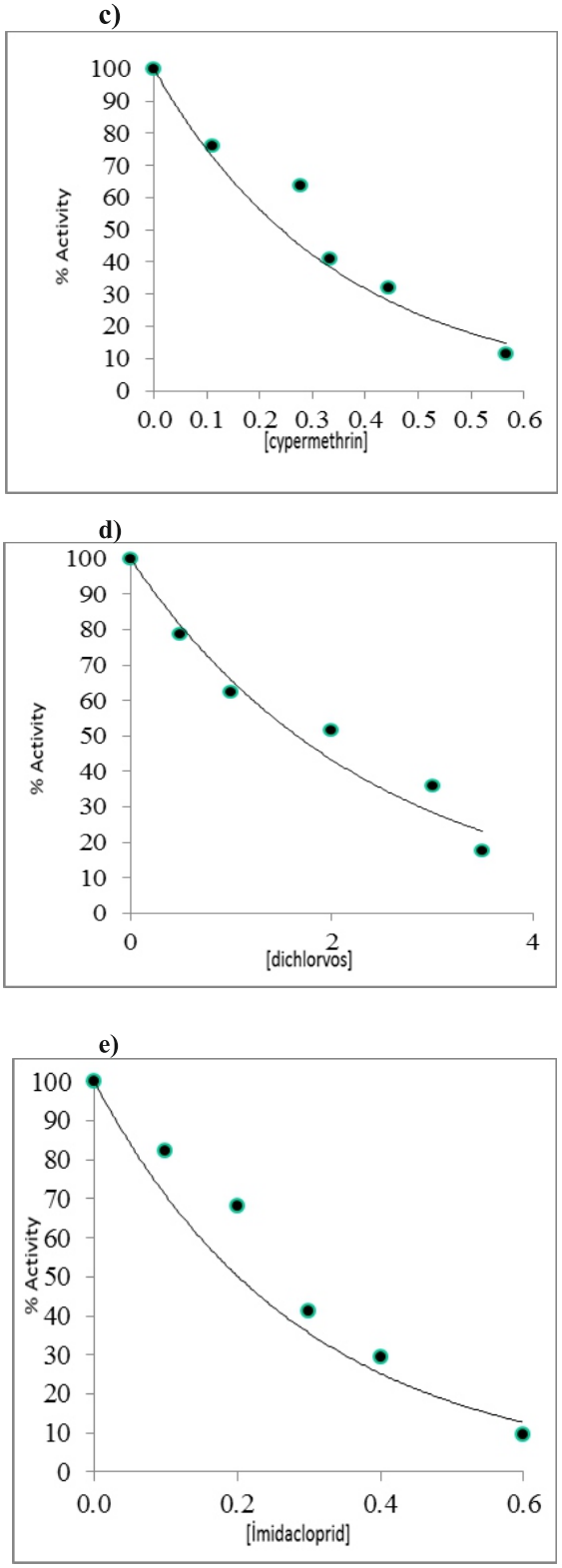


Figure 6. IC_{50} values were obtained from activity (%) – pesticide concentration graph (fig.6a,b,c,d,e).

3. RESULTS AND DISCUSSION

In the present study, GST enzyme was first isolated and characterized from laurel fruit. Purification procedure was carried out by the preparation of the homogenate and Glutathione-Agarose affinity chromatography. As a result of the one consecutive step, the enzyme was purified 30.34 fold with a yield of 50 %, with a specific activity of 0.0058 U/mg from laurel fruit (Table 1). The purity of the enzyme was determined by SDS-PAGE and showed one band on the gel (fig.1). The result indicates that the purification process is sufficient, can be used for further studies and not time-consuming.

The optimum pH for enzyme activity was observed pH 7.0 in 0.1 M K- phosphate buffer and pH 9.0 in 0.1 M Tris- HCl buffer. When enzyme activities were compares against each other, at pH 7.0 in 0.1 M K- phosphate buffer was the optimum pH, and the enzyme catalyzing the reaction at the maximum rate (Fig 2).

Enzyme activites were measured between 0.0625 M and 1.5 M K- phosphate buffer and Tris- HCl concentrations in order to determine effects of ionic strenght on the enzyme activity. Maximum enzyme activity was obtained in 0.125 M K-phosphate buffer that is the optimum ionic strenght of the enzyme activity (fig.3).

To determine the temperature profile of GST, enzyme activities were measured the between 0 and 75 °C. According to the present results the optimum temperature for enzymatic activity was at 30 °C. Enzyme activity increases constantly up to 30 °C, and than exhibits maximum activity at 30 °C that is the optimum temperature for enzymatic activity. However, activity decreases sharply after 30 °C (fig.4).

The kinetic parameters, Km and Vmax were determined using GSH and CDNB as co-substrates. Km values were calculated as 0.194 and 0.353 mM for, and also Vmax values were found as 0.038 and 0.099 EU/ml for CDNB and GSH, respectively (Table 2). According to our result, it can be said that the affinity of GSH is better than CDNB to enzyme as a substrate. Similar results have been showed as follows; in human erythrocytes [29], bovine erythrocytes [30].

4. CONCLUSIONS

Food can be contaminated by microbial or environmental reasons. Environmental contaminants are pesticides, heavy metals, industry wastes and other chemical agents that are present in the environment where the food grown, harvested, transported, stored, and packaged. In this way, poultry, vegetables, dairy and meat products are commonly containing pesticides such as diklorvos, cypermethrin, imidacloprid, fenoxaprop-p-ethyl, glyphosate isopropilamine salt that can enter the food supply chain, ingested by humans try as part of the diet. The findings from this study to make people aware of the effects of pesticides on laurel fruit GST enzyme activity with these obtained K_i and IC_{50} values, thus laurel fruits producer and consumer will be more careful.

Table 1. Purification of GST from laurel fruit.

Purification Step	Total Volume (ml)	Activity (U/ml)	Total Activity (U)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification Factor
Homogenate	13	0.128	1.664	286.78	390	0,0058	100	1
Affinity chromatography	8	0.104	0.832	4.72	6.4	0.176	50	30.34

Table 2. V_{max} and K_M values of laurel fruit GST

Substrate	V_{max} (EU/ml)	K_M (mM)
CDNB	0.099	0.353
GSH	0.038	0.194

REFERENCES

[1]. Ayaz, F.A., Reunanen, M., Küçükislamoğlu, M.M., Seed Fatty Acid Composition in Wild Form and Cultivars of Lauorocerasus Officinalis Roem, *Pakistan J. Bot.* 276 (2), 305-308, 1995.

[2]. Baytop, T., Türkiye'de Zehirli Bitkiler, Bitki Zehirlenmeleri ve Tedavi Yöntemleri; Yayin 3560; Istanbul Üniversitesi Yayinları: Istanbul, Turkey, 1989.

[3]. Milan, S.P.A., The Macdonald Encyclopedia of Medicinal Plants; Macdonald: London, U.K., 1984; p 252.

[4]. Ayaz, F.A., Studies on Water Soluble Sugar and Sugar Alcohol in Cultivar and Wild Forms of Laurocerasus Officinalis Roem, *Pakistan J. Bot*., 29 (2), 331-336, 1997.

[5]. Robards, K., Prenzlere, P.D., Tucker, G., Swatsitang, P., Glover, W., Phenolic Compounds and Their Role in Oxidative Processes in Fruits, *Food Chem.*, 66, 401-436, 1999.

[6]. Vaya, J., Belinky, P.A., Aviram, M., AntioxidantCconstituents from Licorice Roots: Isolation, Structure Elucidation and Antioxidative Capacity Toward LDL Oxidation, *Free Radical Biol. Med.,* 23 (2), 302-313, 1997.

[7]. Vinson, J.A., Su, X., Zubik L., Bose, P., Phenol Antioxidant Quality in Food: Fruits, *J. Agric. Food Chem.*, 49, 5315-5321, 2001.

[8]. Smirnoff, N., The Function and Metabolism of Ascorbic Acid in Plants. Ann. Bot. London, 78, 661-669, 1996.

[9]. Storz, G., Imlay, J., Oxidative Stress, *Curr. Opin. Microbiol*., 2, 188-194, 1999.

[10]. Ulubelen, A., Topçu, G., Tan, N., Ölçal, S., Johanson, C., Üçer, M., Birman, H., Tamer, S., Biological Activities of a Turkish Medicinal Plant, Prangos Platychlaena, *J. Ethnopharmacol.*, 45(3), 193-197, 1995.

[11]. Yan, X., Murphy, B.T., Hammond, G.B., Vinson, J.A., Neto, C.C., Antioxidant Activities and Antitumor Screening of Extracts from Cranberry Fruit (Vaccinium Macrocarpon), *J. Agric. Food Chem*., 50, 5844-5846, 2002.

[12]. Ranson, H., Rossiter, L., Ortelli, F., Jensen, B., Wang, X.L., Roth, C.W., Collins, F.H., and Hemingway, J., Identification of a Novel Class of Insect Glutathione S-Transferases Involved in Resistance to ddt in the Malaria Vector Anopheles gambiae, *Biochem. J.*, 359, 295-304, 2001.

[13]. Lumjuan, N., Rajatıleka, S., Changsom, D., Wıcheer, J., Leelapat, P., Prapanthadara, L., Somboon, P., Lycett, G., and Ranson, H., The Role of the Aedes Ring Resistance to DDT and Pyrethroid Insectidies, *Insect. Biochem. Mol. Biol.,* 41, 203-209, 2011.

[14]. Furtado, A.F., Magalhaes, T., Macoris, M.L.G., and Righetti, M.T M., and Ayres, C.F.J., Resistance to the Organophosphate Temephos: Mechanisms, Evolution and Reversion in an Aedes Aegypti Laboratory Strain from Brazil. Acta. Trop., 113, 180-189, 2010.

[15]. Low, W.Y., S.C., Ng, H.L., Gorman, M.A., Morton, C. J., Pyke, J., Mecconville, M.J., Bieri, M., Mok, Y.F., Robin, C., Gooley, P R., Parker, M.W., and Batterham, P., Recognotion and Detoxification of the Insecticide DDT by Drosophila Melanogaster Glutathione S-Transferase D1, *J. Mol. Biol.,* 399, 358-366, 2010.

[16]. Clark, A.G., The Glutathione S-trasferases and Resistance to Insecticides, pp. 369-378 In J. D. Hayes, C.B., Pickett and T.J., Mantle [eds.]. Glutathione S-transferases and Drug Resistance. Proc. 3rd Int. GST Conferance, Royal College of Physicians, Edinburgh, Scotland, UK, 20-30 Aug 1989.

[17]. Fukami, J.I., and Shishido, T., Nature of a Soluble Glutathione-Dependent Enzyme System Active in Cleavage of Methyl Parathion to Desmethyl Parathion, *J. Econ. Entomol*., 59, 1338-1346, 1996.

[18]. Edwards R., Dixon D.P., Walbot, V., Plant Glutathione S-transferases: Enzymes with Multiple Functions in Sickness and in Health, *Trends Plant Sci.*, 5, 193-198, 2000.

[19]. Dixon D.P., Davis, B., Edwards, R., Functional Divergence in the Glutathione Transferase Superfamily in Plants, *J. Biol. Chem.,* 277, 30859-30869, 2002.

[20]. Banerjee, S., Goswami, R., GST Profile Expression Study in Some Selected Plants: In Silico Approach, *Mol. Cell Biochem.,* 336, 109-126, 2010.

[21]. Soranzo, N., Sari Gorla, M., Mizzi, L., De Toma, G., Frova, C., Organisation and Structural Evolution of the Rice Glutathione S-Transferase Gene Family, *Mol Genet. Genom.*, 271, 511-521, 2004.

[22]. Güvercin, S., Erat, M., Şakiroğlu, H., *Protein & Peptide Letters,* 15, 6-12, 2008.

[23]. Akkemik, E., Taser, P., Bayindir, A., Budak, H., Ciftci, M., *Environmental Toxicology an Pharmacology*, 34, 888-894, 2012.

[24]. Chun-hua, Z., Ze-ying, W., Ting, J., Ying, G., *Rice Science*, 20(3), 173-178, 2013.

[25]. Toriba, F., Martinez- Lara, E., Pascual, P., and Lopez-Barea, J., *J. Chromatogr. B.,* 684, 77, 1996.

[26]. Simons, P.C., and Vander Jagt, D.L., *Methods Enzymol*., 77, 235, 1981.

[27]. Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S- transferases. The first enzymmatic step in mercapturic acid formation. J Biol Chem, 246, 7130-7139.

[28]. Bradford M.A. Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem.,* 72, 248-254, 1976.

[29]. Awasthi, Y.C., Singh, S.V., Purification and Characterization of a New form of Glutathione S-Transferase from Human-Erythrocytes. *Biochem. Biophys. Res. Commun,* 125, 1053-1060, 1984.

[30]. Demirdag, R., Yerlikaya, E., Kufrevioglu, O.I., Gundogdu C., Purification of Glutathione S-transferase Isoenzymes from Tumour and Nontumour Human Stomach and Inhibitory Effects of Some Heavy Metals on Enzymes Activities, *Journal of Enzyme Inhibition and Medicinal Chemistry*, 28(5), 911-915, 2013.