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RESEARCH ARTICLE

Inhibitory Effect of L-Canavanine and L-Lysine on Arginase Activity in Sheep Spleen Tissue [1]

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Abstract

Arginase (L-arginine amidinohydrolase; (E.C.3.5.3.1) is the last enzyme of urea cycle and it catalyzes the hydrolysis of L-arginine to urea and L-ornithine in livers of animals. It helps to the excretion process of urea from the body, which is the most soluble and non-toxic form of nitrogen. Along with its essential role in urea synthesis, arginase is also found to have important roles in ornithine production for polyamine, proline and glutamate synthesis and in immune system activation. Mostly found in liver tissue, arginase enzyme is also found in non-ureolytic tissues like heart, rumen, skeletal muscle, kidney, intestine, brain, spleen, thyroid gland, salivary gland, erythrocyte, fibroblast, macrophage, mammary gland in lactation, testicle. With this study, it was aimed to assess the inhibitory effects of arginase in sheep spleen tissue on L-canavanine and L-lysine. Consequently, it was observed that L-lysine as a L-amino acid and guanidine compound L-canavanine inhibits enzyme activity non-competitively.

Keywords: *Arginase, L-lysine, L-canavanine, Sheep spleen tissue*

L-Kanavanin ve L-Lizin'in Koyun Dalak Doku Arginaz Aktivitesi Üzerine İnhibisyon Etkisi

Özet

Arginaz (L-arginin amidino hidrolaz; (E.C.3.5.3.1) üre döngüsünün son enzimi olup hayvanların karaciğerlerindeki L-arginini, üreye L-ornitine hidrolizini katalizeleyen bir enzimdir. Azotun en iyi çözünebilir ve nontoksik formu olan üre oluşumuna katkıda bulunarak vücuttan atılmasına yardımcı olur. Arginaz üre sentezindeki vazgeçilmez rolünün yanında; poliamin, prolin ve glutamat sentezi için ornitin üretimini sağlayıp, ayrıca immün sistem aktivasyonunda ve tümör biyolojisinde de rol oynadığı saptanmıştır. Arginaz enzimi en çok karaciğer dokusunda bulunmakla beraber; kalp, rumen, iskelet kası, böbrek, bağırsak, beyin, dalak, tiroit bezi, tükürük bezi, eritrosit, fibrobast, makrofaj, laktasyondaki meme bezi, testis gibi nonüreolitik dokularda da bulunmaktadır. Bu çalışma ile koyun dalak doku arginazı üzerine L-kanavanin ve L-lizinin inhibisyon etkileri incelenmesi amaçlanmıştır. Sonuç olarak, bir L-amino asit olan L-lizin ve guanidin bileşiklerinden L-kanavaninin enzimi non-kompetetif olarak inhibe ettiği görüldü.

Anahtar sözcükler: *Arginaz, L-lizin, L-kanavanin, Koyun dalak dokusu*

INTRODUCTION

Arginase enzyme (E.C.3.5.3.1), L-arginine amidinohydrolase), discovered by Kossel and Dakin in 1904, is the last enzyme of urea cycle [1]. In mammalians, there are two isoforms of arginase that hydrolyze L-arginine to ornithine and urea. Arginase I is the cytoplasmic enzyme of liver. Arginase II is found in mitochondria of extrahepatic tissues such as brain, small intestine, and macrophages [2].

Liver, in which urea cycle takes place, contains most of the arginase in the body. Arginase is also found in extrahepatic tissues with no urea synthesis such as kidney, brain, thyroid gland, salivary gland, erythrocytes, thrombocytes, macrophages, rumen, mammary gland, skeletal muscle, fibroblast, intestine, uterus and testicle [3-7]. It is known that arginase provides the necessary ornithine

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for proline, glutamate, and polyamine synthesis in these tissues with no urea cycle [8].

Arginase enzyme is the first enzyme of polyamine biosynthesis. Polyamines (putrescine, spermine, and spermidine) are important molecules for cell growth and differentiation. Ornithine, formed with the help of arginase, is then converted to putrescine by ornithine decarboxylase enzyme; and later putrescine joins in the synthesis of spermine and spermidine [9,10].

Studies showed that serum arginase activity increases in liver diseases, such as acute hepatitis, liver metastases, malignant tumors of bile duct, and cirrhosis, which cause cell destruction [11].

In different studies, it is stated that arginase is a determinant enzyme in cancer processes, because serum and tissue arginase levels increase in some cancer cases [11-13].

Serum arginase levels of patients with acute and chronic pancreatitis were found higher than control values and arginase activity was decreased after treatment [14].

Increase in erythrocyte arginase levels was found in led poisoning and diseases like pernicious anemia and thalassemia [15].

It is stated that in patients with myocardial infarction, arginase levels were increased in the first day and in the following day, these levels were a little lower than the levels of the first day, but were still higher than the control values. It was observed that in 10^{th} day arginase levels returned back normal [16].

Ceylan *et al.*[17], investigated plasma arginase levels in the blood samples taken from asthma patients and found that arginase activities were lower than the control values.

It is found that arginase enzyme exists in some uricotelic organisms and plants [18,19].

It is observed that urea cycle enzymes decrease in low protein diet whereas urea cycle enzyme activities increase in contrast to that of the ones in low protein diet when extra protein is added [20]. It is reported that L-ornithine and L-lysine cause non-competitive inhibition over bovine lumen tissue arginase [21,22]. In view of these findings, we suppose that L-lysine and L-canavanine will inhibit the enzyme. Therefore, in this study, we aim to find the type of the inhibition. To the best knowledge of the authors, this is the first study in the literature which presents the results of kinetic effect of inhibition of L-canavanine and L-lysine on arginase level in sheep spleen tissue, which was not studied before.

MATERIAL and METHODS

Chemicals used in this study, such as L-arginine, diacetyl-monoxyme, thiosemicarbazide, sulfuric acid, HCl, L-lysine, L-canavanine, KCl were obtained from the companies Sigma, Merck and Fluka. Other chemicals used in this study were obtained from the market and in analytical purity.

Sheep spleens as study materials were obtained from Elazığ Elkas Facilities. 20 sheep from the same herd of Akkaraman race with 2 to 3 years old and with similar breeding conditions (shelter, caring and environmental conditions) and similar feeding conditions were slaughtered.

After slaughtering, the obtained spleen tissue was taken into beaker with isotonic cold NaCl solution, after cleaning out from blood and clots; and it was analyzed in iced setting out of the beaker. The rest was kept in deep freezer in -18°C for later use.

After tissue samples were cleaned out from blood and clots, they were weighed as 1 g (weight/volume) and completed to 6 ml with distilled water (dilution rate 1/6). Tissues were homogenized with Potter-Elvehjem homogenizer. The homogenates were processed in +4°C using cold centrifuge (Nuve) device for 14 min and supernatants and pllets were seperated from one another. Obtained supernatant was used as enzyme source.

Arginase activity was measured with spectrophotometry using thiosemicarbazide-diacetylmonoxyme urea (TDMU) method. This method is based on the detection of urea produced by hydrolysis of L-arginine [23].

Diacetylmonoxyme is a substance that does not directly react with urea and hydrolyzes to diacetyl and hydroxylamine in acidic setting with temperature. After diacetylmonoxyme is degraded to diacetyl, diacetylic acid condensates with urea in solution and composes a yellow compound named diazine. This yellow color is stabilized with thiosemicarbazide and Fe^{+2} ions [24].

Protein quantity in the homogenate was measured with modified Lowry [25] method. Alkaline copper tartrate reagent forms complexes with peptide bonds. When phenol reagent is added to mixture, which is processed with copper, a color of violet purple appears. This color was assessed in 650 nm in spectrophotometry.

While measuring the arginase levels, control was established by using triple tubes. For degradation of endogenous urea, 3 units of Jack-Bean urease were added to each ml of supernatant. Then, incubation was done in 37°C for 15 min [26]. Samples were diluted with 2 mM $MnCl_2$ solution with a rate of 1x40 (v/v) and held in metabolic water bath in 58°C for 13 min. Pre-incubation procedure was done the same way and samples were used as enzyme sources.

Enzyme source, which first 120 mM of L-arginine, then

200 mM of carbonate buffer were added to, was pre-incubated. Then, 0.3 ml of enzyme source was added to each tube and enzymatic reaction was initiated in shaking metabolic water bath in (pH 9.5) 37°C for 10 min. After 10 min of incubation, enzymatic reaction in each tube was stopped with 3 ml of acid mixture. Then, 2 ml of color reagent was added to each tube. Tubes were mixed with vortex, then held in boiling water bath for 10 min and color formation was implemented.

Tubes were cooled and assessed in spectrophotometry (Shimadzu UV-240) in 520 nm of wave length. Urea levels were assessed after absorbance values of zero time blanks were subtracted. These procedures were also carried out for standard study tubes and 0 to 0.6 µmol of urea standard solutions were used instead of enzyme sources and then calibration curves were drawn.

An enzyme quantity that produces 1 µmol of urea from L-arginine in 37°C in 1 h is called 1 unit of enzyme, while specific activity is stated as urea/hour/mg protein.

RESULTS

Inhibitory effect of L-lysine on sheep spleen tissue arginase activity in concentrations up to 20 mM was investigated. It was observed that 17% of enzyme activity was lost in 5 mM of L-lysine concentration, 28% of enzyme activity was lost in 10 mM of L-lysine concentration, and 36% of enzyme activity was lost in 15 mM of L-lysine concentration *(Fig. 1)*.

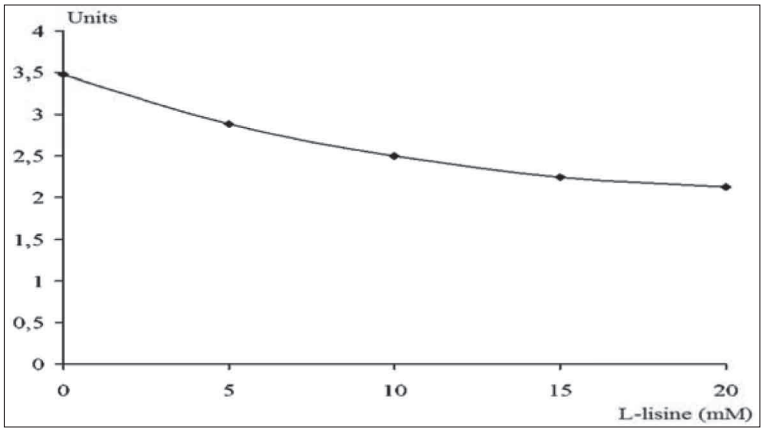
In the presence of 15 mM of L-lysine and in different L-arginine concentrations, inhibition type on sheep spleen tissue arginase activity was determined. Data was assessed with Michaelis-Menten *(Fig. 2)* and Lineweaver-Burk *(Fig. 3)* curves and inhibition type was found as non-competitive.

To assess the effect of L-canavanine on sheep spleen tissue arginase activity, 0 to 0.6 mM of L-canavanine was added to pre-incubation setting and findings were evaluated in comparison to the control. It was observed that 18% of enzyme activity was lost in 0.1 mM of canavanine concentration, 38% of enzyme activity was lost in 0.3 mM of canavanine concentration, 89% of enzyme activity was lost in 0.5 mM of canavanine concentration, and 100% of enzyme activity was lost in 0.6 mM of canavanine concentration *(Fig. 4)*.

To find out the inhibition type of L-canavanine on sheep spleen tissue arginase activity, enzyme activity was investigated in different arginine concentrations and in the presence of 0.45 mM of L-canavanine. Data was assessed with Michaelis-Menten *(Fig. 5)* and Lineweaver-Burk *(Fig. 6)* curves and it was found that L-canavanine inhibits sheep spleen tissue arginase activity non-competitively.

Fig 1. Inhibitory effect of L- lysine on sheep spleen tissue arginase activity

Şekil 1. L-lizinin koyun dalak doku arginazı üzerindeki inhibitör etkisi



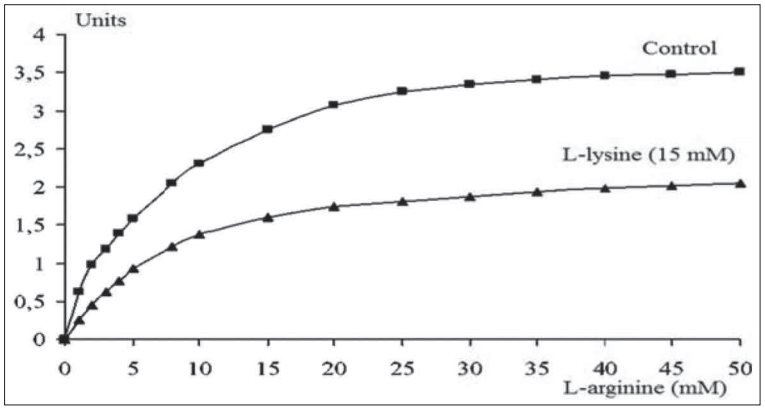


Fig 2. Presentation of inhibitory effect of L-lysine on sheep spleen tissue arginase activity with Michaelis-Menten graph

Şekil 2. L-lizinin koyun dalak doku arginazı üzerindeki inhibitör etkisinin Michaelis-Menten grafiği ile gösterilmesi

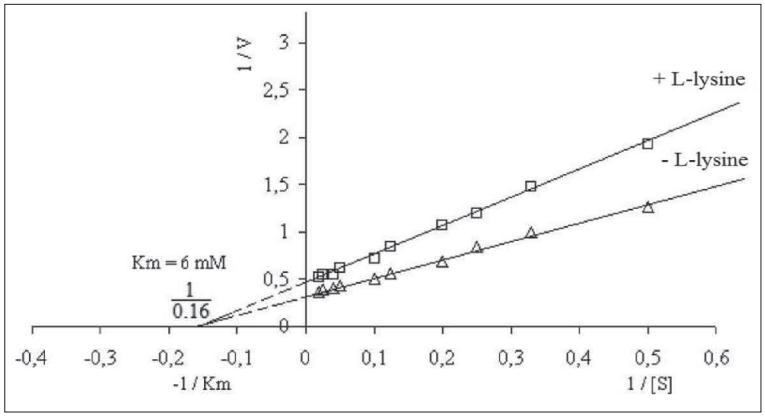
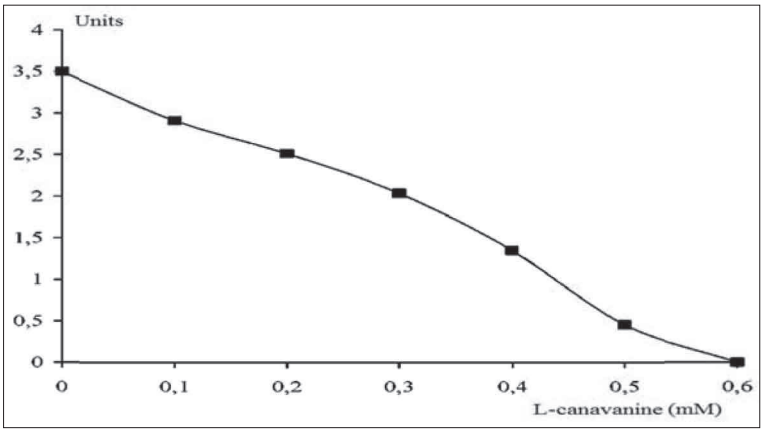


Fig 3. Assessment of effect of L-lysine on sheep spleen tissue arginase activity with Lineweaver-Burk curve

Şekil 3. L-lizinin koyun dalak dokusu arginazı üzerindeki etkisinin Lineweaver-Burk eğrisi ile gösterilmesi

Fig 4. Inhibitory effect of L-canavanine on sheep spleen tissue arginase activity

Şekil 4. L-kanavanin'in koyun dalak doku arginazı üzerindeki inhibitör etkisi



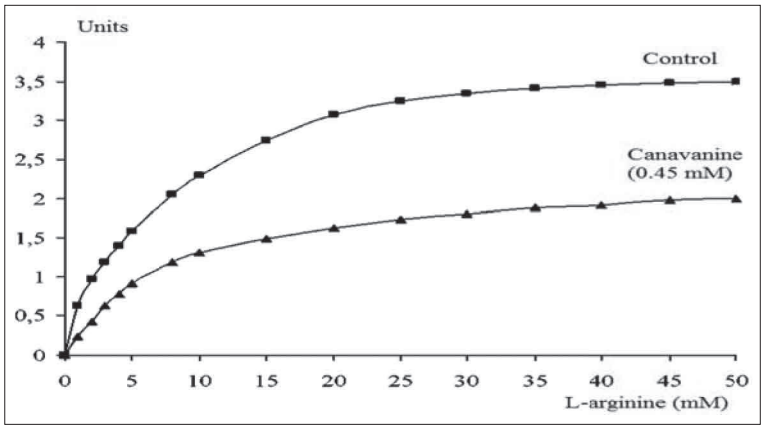
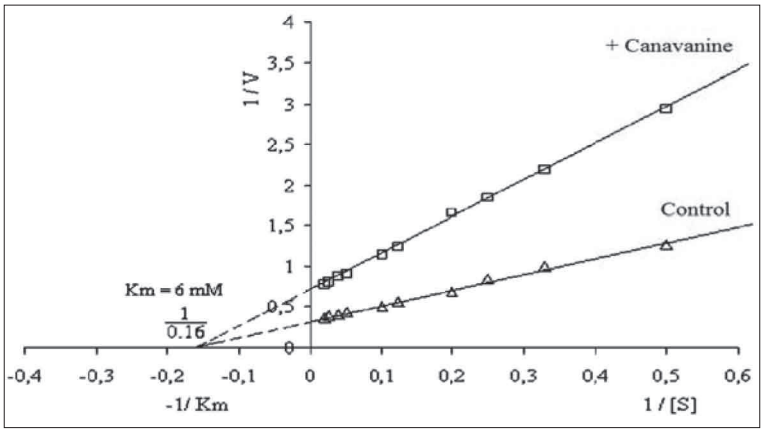


Fig 5. Presentation of inhibitory effect of L-canavanine on sheep spleen tissue arginase activity with Michaelis-Menten graph

Şekil 5. L-kanavaninin koyun dalak doku arginazı üzerindeki inhibitör etkisinin Michaelis-Menten grafiği ile gösterilmesi

Fig 6. Presentation of inhibitory effect of L-canavanine on sheep spleen tissue arginase activity with Lineweaver-Burk curve

Şekil 6. L-kanavaninin koyun dalak doku arginazı üzerindeki inhibitör etkisinin Lineweaver-Burk eğrisi ile gösterilmesi



DISCUSSION

There are a number of inhibitors of the enzyme arginase. Particularly, inhibition of arginase activity with branched chained aminoacids is found in several tissues and species [27,28].

In the studies by Muszynska [29] and Muszynska and Wojtczak [30] it was found out that ornithine and lysine competitively; valine, leucine, isoleucine and cysteine non-competitively inhibit arginase in rat liver.

It is reported that L-aminoacids such as valine, leucine, isoleucine, ornithine, proline, and cysteine inhibit arginase in bovine rumen tissue [21].

Several studies revealed that ornithine and lysine inhibit arginase in mammary gland [31] and in bovine kidney tissue [32].

Fuentes JM *et al.*[33] stated that lysine, ornithine, valine strongly and competitively inhibit arginase in rat mammary gland; while proline, isoleucine and leucine cause less inhibition.

It was seen that lysine inhibits arginase in rat liver mitochondria [34]. It was stated that lysine does the best inhibition of arginase among all amino acids and that it inhibits the enzyme in a mixed way [24].

Also, Subrahmanyam and Reddy [35] proved that lysine is a strong inhibitor of the enzyme in bovine liver and that it inhibits the enzyme competitively.

Levillain *et al.*[36] investigated inhibitory effects of lysine, proline, ornithine and glutamine in different parts of kidney tubules of ducks and stated that the most powerful inhibitor of arginase in kidney tubules is lysine and that ornithine and glutamine come after lysine, while proline had no statistically significant inhibitory power.

In studies on human erythrocyte arginase, it was stated that lysine is a competitive inhibitor and canavine is a non-competitive inhibitor [37].

In an other study [38], 0.5 mmol/kg of L-lysine was injected to children between 6 to 14 years of age; and then, increases in plasma ornithine and arginine levels and urine ammonia concentration were detected. This case was explained with the inhibition of the enzyme arginase. It was suggested that this increase in plasma ornithine was caused by inhibition of mitochondrial ornithine transport. The study concluded that lysine may inhibit both ornithine transcarbamylase activity and mitochondrial ornithine uptake, preventing the conversion of ornithine to citrulline.

In this study, inhibitory effect of L-lysine as an L-amino acid on sheep spleen tissue arginase activity was investigated and it was found that L-lysine inhibits the enzyme. Inhibition type was assessed with Michaelis-Menten and Lineweaver-Burk curves and non-competitive inhibition of the enzyme was observed.

As a substrate for purified human liver arginase, L-canavanine was used instead of L-arginine and an increase in Km from 10.5 mM to 50 mM was observed by Berüter *et al.*[39].

Non-competitive inhibition of liver arginase by canavanine was found; however, it was found that canavanine did not affect arginase in erythrocyte and uterus and it was reported that canavanine can not be used as a substrate instead of arginine [40].

In another study, Muszynska and Wojtczak [30] stated that they could not detect bovine liver arginase activity when canavanine was used, but they detected a low level of arginase activity in chicken liver.

In this study, effect of L-canavanine, as one of the guanidino compounds, on sheep spleen tissue arginase activity was investigated and non-competitive inhibition of the enzyme by L-canavanine was found.

It was suggested that due to the structural similarity between canavanine and arginase, it binds to a non-active part of the enzyme and changes its three-dimensional structure [21].

It is observed that arginase enzyme level varies with respect to nutrition and hormone level. It is reported that arginase enzyme level also varies due to protein intake diet and this variation is not enzyme kinetics, it is the increase in the amount of the enzyme molecules [20].

Studies show that, urea cycle enzymes decrease in low protein diet. When more proteins are added to the diet, it is observed that the urea cycle enzyme activities increase compared to those in low protein diet [22]. In this study, effect of L-lysine aminoacid and L-canavanine on arginase enzyme, which has a prominent role in urea cycle is studied.

To the knowledge of authors no study on kinetic properties of arginase enzyme in sheep spleen tissue has been conducted. We hope that this new data will help for future studies on this issue.

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