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RESEARCH ARTICLE

The Effect of Dietary Antioxidants on the Arginase Activity and Nitric Oxide Level of Freshwater Crayfish (*Astacus leptodactylus*, Esch. 1823)

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Summary

The effects of dietary antioxidants (vitamin E (VE), vitamin C (VC), vitamin A (VA), astaxhantin (ASX), β-carotene (βC)) on the arginase activity and nitric oxide (NO) level of the hepatopancreas, muscle, ovarian and gills of freshwater crayfish *(Astacus leptodactylus)* were investigated. In the investigation, 150 mg kg^{-1} VE, 200 mg kg^{-1} VC, 240 mg kg^{-1} VA, 200 mg kg^{-1} ASX and 200 mg kg^{-1} βC were added to experimental diets. The study was carried out for 144 days. The arginase activity in the hepatopancreas were significantly higher in the VE, VC and VA diet groups in the comparison to control ($P<0.001$), but NO level was lower in all diet groups compared to the control ($P<0.001$). Arginase activity and NO level in the muscle were significantly higher in the some diets groups (arginase; VE, VA, ASX, βC, NO; VC, ASX) according to control ($P<0.001$). The arginase activity and NO levels in the ovarian were significantly lower in the all diet groups in the comparison to control ($P<0.001$). However, no significant differences in the arginase activity and NO level in the gill were observed among diets. The arginase activity and NO level in the hepatopancreas, muscle and ovarian were changed by dietary supplement of the antioxidants.

Keywords: *Astacus leptodactylus, Arginase, Nitric oxide, Antioxidants*

Tatlısu Istakozu (*Astacus leptodactylus*, Esch. 1823)'nun Arginaz Aktivitesi ve Nitrik Oksit Seviyesi Üzerine Rasyon Antioksidanlarının Etkisi

Özet

Tatlısu istakozu *(Astacus leptodactylus)* rasyonuna antioksidan (vitamin E (VE), vitamin C (VC), vitamin A (VA), astaksantin (ASX), β-caroten (βC)) ilavesinin hepatopankreas, kas, ovaryum ve solungaç arginaz aktivitesi ve nitrik oksit seviyesi üzerine etkisi araştırılmıştır. Deneysel rasyonların hazırlanması için gruplara 150 mg kg^{-1} VE, 200 mg kg^{-1} VC, 240 mg kg^{-1} VA, 200 mg kg^{-1} ASX ve 200 mg kg^{-1} βC ilave edildi. Çalışma 144 gün sürdürüldü. Hepatopankreasdaki arginaz aktivitesi kontrol ile karşılaştırıldığında VE, VC ve VA rasyon gruplarında önemli derecede daha yüksekti ($P<0.001$), fakat NO seviyesi kontrol ile karşılaştırılan bütün rasyon gruplarında daha düşüktü ($P<0.001$). Kasdaki arginaz aktivitesi ve NO seviyesi bazı rasyon gruplarında (arginaz; VE, VA, ASX, βC, NO; VC, ASX) önemli derecede daha yüksekti ($P<0.001$). Ovaryumdaki arginaz aktivitesi ve NO seviyesi kontrol ile karşılaştırıldığında bütün rasyon gruplarında önemli derecede daha düşüktü ($P<0.001$). Ayrıca solungaçlardaki arginaz aktivitesi ve NO seviyesinde rasyonlar arasında hiçbir farklılık görülmedi. Hepatopankreas, kas ve ovaryumdaki arginaz aktivitesi ve NO seviyesi antioksidanların rasyona ilave edilmesi ile değişti.

Anahtar sözcükler: *Astacus leptodactylus, Arginaz, Nitrik oksit, Antioksidan*

INTRODUCTION

Astacus leptodactylus is a commercial species in Keban Dam Lake in the east of Turkey. This species has also commercial importance in Turkey and until 1986 was exported to a number of European countries. The production of *A. leptodactylus* after 1985 decreased dramatically in most Turkish lakes (from 5000 tones annually to 200 tones) as a result of the crayfish plague *(Aphanomyces astaci),* overfishing, pollution, and water extraction for agricultural irrigation [1].

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Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) catalyses the hydrolysis of L-arginine to form L-ornithine and urea in the final reaction of the urea cycle [2]. Arginase enzyme is also found in organs and organisms which do not synthesize urea [3,4]. Although crayfish are an ammoniotelic organism and do not contain an active urea cycle, it has also an active arginase enzyme [5-8]. Arginase is a key enzyme of the intermediary metabolism. It is directly involved in the protein metabolism, in the synthesis of creatinine, nitric oxide, polyamines and proline/glutamate [9].

Nitric oxide, NO, is generated from L-arginine by the nitric oxide synthase family of enzymes [10]. NO signaling is involved in many physiological processes in invertebrates. In crustaceans, it plays a role in the regulation of the nervous system and muscle contraction [11]. However, the reaction of nitric oxide (NO) and $O_2^{\cdot-}$, which is produced by nitric oxide synthetases, can form reactive peroxynitrite, which rapidly breaks down into $^{\cdot}OH$ and nitrogen dioxide radical [12]. NO, which is a highly reactive free radical, damages proteins, carbohydrates, nucleotides and lipids and, together with other inflammatory mediators, results in cell and tissue damage. NO acts in a variety of tissues to regulate a diverse range of physiological processes, but excess of NO can be toxic [13].

Vitamin E (VE), vitamin C (VC), vitamin A (VA), astaxanthin (ASX) and β-carotene (βC) have been supplemented to diet to increase the reproduction, to modulate the antioxidant defense system and to provide the optimum growth in aquatic organism [1,14-18]. Apart from this, carotenoids are used to pigment the muscle of farmed fish and crustacean [16].

Like other aquatic organism, the interaction among arginase activity, NO level and antioxidants of crayfish have yet to be investigated. The effects of different levels of dietary vitamin E on the arginase activity of the tissues of Astacus leptodactylus were investigated by Erişir [2].

The aim of this study was to evaluate the effects of antioxidants (VE, VC, VA, ASX and βC) supplemented diet feed up to spawning period on arginase activity and NO level of the hepatopancreas, ovarian, muscle and gills of *A. leptodactylus*.

MATERIAL and METHODS

Experimental Protocol

This study was carried out between August 15, 2007 and January 06, 2008 at the aquarium laboratory of Fırat University Aquaculture Faculty, Elazığ, Turkey. The crayfish used in the present study was provided from Keban Dam Lake population of *A. leptodactylus.*

Crayfish were housed in 18 glass aquariums (25x25x110 cm). Plastic pipes (15 cm in length and 7 cm in diameter) were provided as shelters for the crayfish. Adequate aeration was provided for each aquarium by a simple air pump. *A. leptodactylus* were acclimatised to temperature and flow conditions and starved for one week to standardize their nutritional conditions and to ensure that they were in good health prior to the start of the experiment. Triplicate groups of crayfish (12 individuals per group) were randomly assigned to each feeding treatment on August 22. The carapace length (mm) and weight (g) were recorded for each crayfish. Crayfish were fed 2% of their total wet weight daily, divided into three separate feedings [1]. After 137 days, a sample of 9 crayfish from each of the six dietary treatments was randomly selected for analysis. For biochemical assays, the hepatopancreas, muscle, gonad and gills in the crayfish were removed and were stored at -80°C until used.

During the trial, mean dissolved oxygen 6.67±0.29 mg/L; mean pH was 7.89±0.8 and water temperature were 17.45±1.27°C.

Experimental Diets

The VE, VC, VA, ASX and βC contents of the diets were analyzed by High Performance Liquid Chromatography [19-20]. The crude protein content was analyzed by Kjeldahl's method; the Gross energy was calculated based on physiological fuel values of 9 kcal/g for lipid and 4 kcal/g for protein and carbohydrate; the dry matter was determined after the sample was dried at 105ºC for 6 h; the ash content was determined after 24 h at 550ºC in the furnace; the lipid was analyzed by an ether extraction method [21].

The practical control diet used in this study *(Table 1)* was modified after Harlıoğlu [1] and Barım [22]. The control diet was formulated to contain approximately 38.86% crude protein on a dry-weight basis and 3.32 kcal/g gross energy. Levels of dietary VE (150 mg kg^{-1}) [22], VC (200 mg kg^{-1}) [14,23], VA (240 mg kg^{-1}) [14], ASX (200 mg kg^{-1}) [15,16] and βC (200 mg kg^{-1}) [15,16] were set in relation to levels reported by other researchers in a variety of crustacean species. The VE, VC, VA, ASX and βC contents of the control were 11.71±1.27 mg kg^{-1},

14.25±1.23 mg kg^{-1}, 2.21±0.17 mg kg^{-1}, 1.45 mg kg^{-1}, 17.15±0.12 mg kg^{-1}, respectively. Based on analysis, levels of 132.04±1.26 mg kg^{-1} VE, 178.14±2.78 mg kg^{-1} VC, 230.50±1.54 mg kg^{-1} VA, 185.77±2.37 mg kg^{-1} ASX and 174.82±2.14 mg kg^{-1} βC diet were determined for VE, VC, VA, ASX and βC diets, respectively. No VE, VC, VA, ASX and βC was added to the control diet, except that supplied by the feed ingredients. Dietary VE (50% dl-α-tocopheryl acetate), VC (33% L-ascorbic acid monophosphate), VA (1000000 IU per gram retinyl acetate), ASX (8% astaxanthin, Carophyll Pink,) and βC (10% β-Carotene) was donated by DSM. The ingredients for each diet were thoroughly mixed, before adding water, in a commercial food mixer, cold-pelleted by forcing through 3 mm holes using a laboratory pellet mill, air-dried at 5ºC for up to 24 h, and then stored in a deep freeze at -20ºC until further use.

Table 1. *Composition and proximate analysis of the control diet*
Table 1. *Kontrol rasyonunun kompozisyonu ve yaklaşık analizi*

Ingredient	Percent of dry weight
Fish (anchovy) meal	35.78
Soybean meal	38.64
Wheat flour	19.30
Sunflower oil	4.00
Dicalcium phosphate	1.00
Sodium phosphate	0.40
Avilamycine [1]	0.10
Antioxidant [2]	0.10
Vitamin E, A, C-free vitamin premix [3]	0.50
Mineral premix [4]	0.18
Proximate composition	
Crude protein	38.86
Crude fat	8.02
Crudefibre	3.02
Crude ash	14.17
Nitrogen free extract	28.93
Moisture	7.00
Gross energy (kcal/g)	3.32

1 *Kavilamycine*
2 *Antioxidant (mg/kg dry diet): butylated hydroxytoluene 12.5*
3 *Vitamin premix (mg/kg): Menadion 600, Riboflavin 1200, Pridoxin 1000, Cobalamin 3, Niacin 5000, Biotin 8, Folic acid 200, Colin clorid 60, Calcium D-Pantothenate 1600 Calsiferol 400000*
4 *Mineral premix (mg/kg dry diet): Mn 80, Fe 35, Zn 50, Cu 5, I 2, Co 0.4, Se 0.15*

Sample Preparation and Biochemical Assays

The tissues were weighed and homogenized with 10 volumes of 10 mM Tris-HCl buffer pH (7.4) in a glass Potter Elvehjem homogenizer in an ice bath. The homogenates were centrifuged at 20.000 g for 10 min at 4ºC. The supernatants were used for the arginase and NO assay.

Arginase Activity Assay: Arginase activity was measured spectrophotometrically in the optimized conditions for crayfish [24] by the thiosemicarbazide diacetylmonoxime urea (TDMU) method of Geyer and Dabich [25] one unit of arginase activity was expressed as the amount of enzyme catalyzing the formation of one mmole of urea h^{-1} at 37ºC. The results are given as units/mg of protein. Protein was measured by the method of Lowry [26] using bovine serum albumin as standard.

NO levels Assay: NO measurement is very difficult in biological specimens, because it is easily oxidized to nitrite (NO_2) and subsequently to nitrate (NO_3) which serve as index parameters of NO production. Samples were initially deproteinized with NaOH and $ZnSO_4$. Total nitrite (NO_2+NO_3) was measured by spectrophotometer at 545 nm after conversion of NO_2 to NO_3 by assay reactive. A standard curve was established by a set of serial dilutions of sodium nitrite. Results were expressed as μmol per gram tissue [27].

Statistical Procedures: Results were expressed as mean±SEM. Analysis of variance (ANOVA) followed by Duncan test was used to determine whether there were significant differences among the groups. The 5% level of significance was used to establish differences.

RESULTS

The carapace length among the experimental groups (VE, VC, VA, ASX, βC and control) and within the replicates of each dietary treatments were not significantly different (P>0.05 for each cases) at the beginning of the experiment. The mean carapace length and weight of crayfish was 48.72±0.64 mm, 27.35±1.00 g for VE, 47.67±0.76 mm, 26.16±1.28 g for VC, 48.97±0.22 mm, 28.08±1.17 g for VA, 47.31±0.66 mm, 26.27±1.07 g for ASX, 49.00±0.71 mm, 27.99±1.23 g for βC, 48.47±0.62 mm, 27.62±0.98 g for control.

Arginase activity and NO levels of hepatopancreas, muscle, gonad and gill tissues were shown in *Table 2*.

The arginase activity in hepatopancreas of crayfish fed the diets VE, VC and VA were significantly higher (315.62%, 687.5%, 165.62% respectively) than those of crayfish fed the control diet, but the NO levels in VE, VC, VA, ASX and βC groups were lower (82.61%, 29.86%, 84.94%, 11.08%, 29.86% respectively). It was found that the concentration of the arginase activity in muscle of crayfish fed the diets VE, VA, ASX and βC were higher than the control (94.01%, 81.695%, 154.92%, 177.46% respectively). Similarly, NO levels

Table 2. *The mean concentrations of arginase (units mg^{-1}) and NO (µmol g^{-1} tissue) in the hepatopancreas, muscle, ovarian, and gills tissue of A. leptodactylus fed on the six diets; Control ©, Vitamin E (VE), Vitamin C (VC), Vitamin A (VA), Astanthin (ASX), βeta carotene (βC)*

Tablo 2. *Altı farklı rasyonla beslenen (Kontrol ©, Vitamin E (VE), Vitamin C (VC), Vitamin A (VA), Astaksantin (ASX), βeta caroten (βC)) A. leptodactylus'un hepatopancreas, kas, ovaryum ve solungaç dokularındaki arginaz (units mg^{-1}) ve nitrik oksitin (µmol g^{-1} tissue) ortalama konsantrasyonları*

Parameters	P						P
	C	VE	VC	VA	ASX	βC	
Hepatopancreas							
Arginase	0.32±0.04 ^{d}	1.33±0.09 ^{b}	2.52±0.13 ^{a}	0.85±0.03 ^{c}	0.48±0.02 ^{d}	0.34±0.05 ^{d}	***
Nitric oxide	15.07±0.13 ^{a}	2.62±0.16 ^{d}	10.57±0.35 ^{c}	2.27±0.09 ^{d}	13.40±0.87 ^{b}	10.57±0.35 ^{c}	***
Muscle							
Arginase	2.84±0.16 ^{c}	5.51±0.45 ^{b}	3.28±0.40 ^{c}	5.16±0.45 ^{b}	7.24±0.13 ^{a}	7.88±0.83 ^{a}	***
Nitric oxide	6.25±0.25 ^{b}	5.32±0.41 ^{b}	14.72±1.15 ^{a}	6.89±0.58 ^{b}	15.66±0.58 ^{a}	5.22±0.15 ^{b}	***
Ovarian							
Arginase	7.45±0.2 3 ^{a}	2.41±0.11 ^{cd}	2.96±0.08 ^{c}	1.72±0.24 ^{d}	3.89±0.14 ^{b}	4.45±0.52 ^{b}	***
Nitric oxide	13.22±0.48 ^{a}	4.51±0.14 ^{c}	3.68±0.23 ^{c}	9.39±0.78 ^{b}	4.26±0.32 ^{c}	4.53±0.12 ^{c}	***
Gills							
Arginase	7.60±0.44	8.05±0.69	8.18±0.42	8.44±0.30	7.37±0.26	8.19±0.42	-
Nitric oxide	12.88±0.82	11.66±0.59	11.81±0.63	13.02±0.86	12.30±0.70	13.41±0.74	-

*Note: -: P>0.05, ***:P<0.001, ± values: standart error of the means*
Values with different superscripts within the same line were statistically significant (P<0.05)

in these tissues of crayfish in VC and ASX group were higher than control (135.52%, 150.56% respectively).

The arginase activity and NO level in the ovarian tissues were significantly lower than those of crayfish fed the control diet. The percentage decrease in ovarian tissues was 67.65 for VE, 60.26 for VC, 76.91 for VA, 47.78 for ASX and 40.27 for βC on the arginase activity, 65.88 for VE, 72.16 for VC, 28.97 for VA, 67.78 for ASX and 65.73 for βC on the NO level. However, in this study was determined that the supplemental antioxidants were not effected the arginase activity and NO level of the gills tissues.

DISCUSSION

In aquatic vertebrates it was established that feeding is an important factor affecting arginase activity [2]. Vitamins are organic compounds required in small quantities in diet of fish and crustacean [28]. Because some food substance, especially VE, VA and carotenoids, cannot be synthesized by crustacean, these substances must be added in food [18]. Several studies indicated that there was a relationship between vitamins and arginase [2,29,30]. Park [29] reported that rats fed a vitamin E supplemented diet for 40 days had lower liver arginase activity than those fed a vitamin E deficient diet. However, John [30] reported that rats fed a vitamin A supplemented diet for 40 days had lower liver arginase activity than those fed a vitamin A deficient diet. It has been found that liver and kidney arginase activity decreased in rat treated with high doses of vitamin A [31,32]. In the present study we found that the arginase activity in ovarian of crayfish fed VE, VC, VA, ASX and βC supplemented diets was significantly decreased.

Erişir [2] reported that in the ovigerous crayfish (Carapace lenth (CL): 54.6-56.3 mm, weight: 42.0-46.6 g) in comparison with the control, arginase activity in the hepatopancreas, muscle, gills were not significantly affected by 150 mg kg^{-1} vitamin E levels in the diet. In present study it was found that the arginase activity in gills of crayfish (during ovarian maturation) (CL: 47.31-49.00 mm, weight: 26.16-28.08 g) fed VE, VC, VA, ASX and βC supplemented diets were not significantly affected, but in the hepatopancreas and muscle were increased. The differences among the result may be due to the different size and reproduction period of crayfish.

To our knowledge, there are no other data in the literature concerning the effect of ASX and βC on arginase activity. In present study, the effect of ASX and βC on arginase activity was parallel to the other vitamins.

Carbamoyl phosphate synthetase and ornithine

transcarbamoylase from urea cycle enzymes have been shown to be absent in crustaceans [8]. In addition, crustaceans do not only have an active arginase enzyme but also the enzymic capacity to convert ornithine (the second reaction product of arginine hydrolysis) to proline [7,8]. Proline is a fundamental structural element of collagen (connective tissue). Animals such as the earthworm, starfish and mussel evolving to use increased amounts of collagen synthesize proline from the ornithine moiety of arginine [8,33]. Our result illustrate that the response to the vitamins of arginase activity is different according to the tissue. The reduction or elevation observed in arginase activity with dietary intake of the vitamins may affect connective tissue formation in these tissues. Likewise, it has been known that the presence of sufficient ascorbic acid (vitamin C), a required cofactor for prolylhydroxylase, thus requires the formation of stable collagen [34].

Arginase catalyzes the conversion of L-arginine to L-ornithine and urea and is capable of limiting NO production by competing for the common NO synthase substrate L-arginine [35]. The decreased NO levels in the hepatopancreas by effect of the vitamins may be due to use L-arginine by the increased arginase activities. The decrease in both arginase activity and NO level in the ovarian tissues may be related to use in protein synthesis of L-arginine in the ovarian because the crayfish was during ovarian maturation. Likewise, accumulation of biochemical components in the maturing ovary has been reported in fish and crustacea [17,18,36]. For example, Palacious [36] found that the level of total protein in mature ovaries of Penaeus vannamei increased.

NO is produced from the conversion of L-arginine to NO and citrulline in the presence of NO synthase in the arginine pathway [37]. The physiological messenger molecule nitric oxide is produced by the endothelium, nerve cells and hemocytes. In crustaceans, it plays a role in neuronal development, immune defense and neuron, skeletal muscle and cardiac muscle regulation [11,38,39]. It has been reported that in crustaceans, decreased NO levels caused to decrease of nervous system functions and motor neurons' reflex response [40,41]. Except the effect of VC and ASX on NO level in the muscle tissue, the other all vitamins may negatively effect the regulation of nervous system by decreasing the NO level in hepatopancreas and ovarian.

Also, NO is one of the gaseous radicals [42]. The NO level in the muscle of crayfish fed VC, ASX supplemented diet were significantly increased. Presence of VC and ASX in diets may cause cell and tissue damage by increasing NO level in the muscle. However, Sullivan [43] reported that in crustaceans, increased levels of NO decreased the rate of neurogenesis.

The arginase activity and NO level in the hepatopancreas, muscle and ovarian was changed by dietary supplement of the vitamins, whereas in the gills was not effected. The physiological significance and damage of these changes have to be analyzed in further experiments.

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