

Evaluation of optimal conditions for arginase activity in streptozotocin induced diabetic rats

M. ERISIR¹, E. ERCEL², S. YILMAZ¹, S. OZAN¹

¹Department of Biochemistry, Veterinary Faculty of Firat University, Elazig, Turkey

²Province Veterinary Control Laboratory, Elazig, Turkey

ABSTRACT: The assay conditions needed to achieve maximal activity of liver and kidney arginase in diabetic and non-diabetic rats were investigated and compared. The physicochemical and kinetic properties of liver arginase in diabetic and control rats were very similar, those of kidney arginase were significantly different. It was found that preincubation temperature (68°C), preincubation period (20 min), optimum pH (10.1) of liver arginase and K_m (3.2) for its substrate, L-arginine, did not change in diabetic and non-diabetic rats. As a consequence of diabetes, the optimum Mn^{2+} concentration for liver arginase only changed from 1 to 2 mM. Although the preincubation temperature and period for activation of kidney arginase in control rats was unnecessary, they were found to be 56°C and 12 min in diabetic rats. The pH profile of arginase in kidney of diabetic rats was different from that of control rats. The K_m value (6.7) of arginase for L-arginine in kidney is unchanged in diabetes whereas a marked decrease in V_{max} was found. Optimum Mn^{2+} concentration (2 mM) for kidney arginase was unchanged in diabetes. The activity of arginase in liver of diabetic animals was higher 1.5 to 1.7 times than that of controls. Diabetes caused an about 53% decrease of arginase activity in kidney of female rats, 26% in that of males. These findings may suggest an idea that encoded arginases by separate gene loci may be affected differently by the pathological and hormonal status.

Keywords: arginase; diabetes; physicochemical and kinetic properties

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycaemia resulting from disorders of insulin secretion or insulin action or both (Pfaffly, 2001). Streptozotocin (STZ) induces diabetes in rats. STZ is specifically toxic to β -cells of the Langerhans islets of the pancreas, which are responsible for the production of insulin (Junod et al., 1967). Insulin dependent diabetes mellitus (IDDM) is characterized by a series of complications that affect many organs. Diabetic patients also suffer from a wide variety of complications due to their diseases, such as atherosclerosis, retinopathy, poor circulation, and liver and kidney problems (Pfaffly, 2001).

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) catalyses the final step in the urea cycle and is therefore present mainly in the liver (Powers and Meister, 1982). Much lower levels of arginase activity are found in other organs such as kidney which does not contain a complete urea cycle (Aminlari and Vaseghi, 1992).

Arginase activity is very sensitive to hormonal influences and rat liver arginase activity is elevated, for example, in diabetic animals and in response to glucocorticoids and glucagon (Husson et al., 1987; Grofte et al., 1998; Upadhyaya et al., 1999). Insulin-dependent diabetes is accompanied by increased food consumption, amino acid metabolism and ratio of blood glucagon to insulin, all of which would tend to increase the activity of the urea cycle. Increased activity of liver arginase was demonstrated in diabetic animals (McLean and Novello, 1965; Baxter and Schofield, 1980; Bond et al., 1983; Failla, 1986; Spolarics and Bond, 1989; Upadhyaya et al., 1999).

Kidney is a major organ involved in diabetic complications. Diabetic nephropathy is one of the serious complications in patients with IDDM (Winegard, 1987).

Arginase activity is Mn^{2+} dependent. For maximum activity, arginase requires preincubation with Mn^{2+} cations (Spector et al., 1982). An increase in

free Mn^{2+} concentration in the liver of streptozotocin-induced diabetic rats was established (Bond et al., 1983, 1986; Failla, 1986). The assay conditions needed to achieve maximum activity of liver arginase in diabetic rats may also differ from those of non-diabetic rats. Therefore, this experiment was designed to investigate whether the assay conditions needed to achieve maximum activity of liver and kidney arginase in diabetic rats might differ from those of non-diabetic rats. In addition, we planned to measure liver and kidney arginase activities in diabetic and control rats after the assay conditions were optimised.

MATERIAL AND METHODS

Thirty-eight adult Wistar-Albino rats weighing on average 180 g were divided into two groups. All animals received standard laboratory food *ad libitum* and were given free access to water. All animals were maintained at a constant temperature (22°C) with a fixed 12:12-h light-dark cycle. In a diabetic group, a dose of 60 mg/kg of STZ was injected intraperitoneally. STZ was dissolved in 1 ml of citrate buffer (0.1 M, pH 4.5) just before use. Control animals received the citrate buffer solution alone. Seven days after STZ injection, blood glucose levels were determined using an Ames glucometer. Rats with blood glucose levels of 250 mg/100 ml or above were considered to be diabetic.

At the end of 9 weeks, control and diabetic rats were decapitated. The liver and kidneys were excised, weighed and homogenized with 10 volumes of 10 mM Tris-HCl buffer pH (7.4) in a glass Potter Elvehjem homogeniser in an ice bath. The homogenates were centrifuged at 20 000 g for 10 min at 4°C. The supernatants were used for the arginase assay. Half of the supernatants from control and diabetic group was pooled to test the optimum assay conditions. Arginase activities were measured in the other half after the assay conditions were optimised.

Arginase activity was measured spectrophotometrically by the thiosemicarbazide diacetylmonoxime urea (TDMU) method of Geyer and Dabich (1971). The principle of arginase activity determination is spectrophotometric measurement of urea produced by hydrolysis of L-arginine by arginase. One unit of arginase activity was expressed as the amount of enzyme catalysing the formation of one μmol of urea/h at 37°C. The results are given as units/mg of protein (specific activity). Protein was

measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistical analysis. The means \pm SEM were calculated from data obtained from groups. Significance between experimental groups was calculated by Student's *t*-test and Mann-Whitney *U*-test. *P*-values < 0.05 were accepted as significant.

RESULTS

Physicochemical and kinetic properties of liver and kidney arginase from diabetic and control rats were compared.

Influence of preincubation temperature on liver and kidney arginases

The samples were preincubated for 10 min at various temperatures in the presence of Mn^{2+} ions. For liver arginase, the optimum preincubation temperature was 68°C in both control and diabetic groups (Figure 1A). The optimum temperature for kidney arginase activity of diabetic rats was 56°C whereas the kidney arginase activity of control rats was unchanged to 60°C and then decreased progressively (Figure 1B).

Influence of preincubation period on liver and kidney arginases

A 20-min preincubation at 68°C increased the enzyme activity approximately 3 times in the liver of both control and diabetic rats. Therefore, for liver arginase, the optimum preincubation period was determined to be 20 min at 68°C (Figure 2A). Kidney arginase activity of diabetic rats was increased approximately twice following a 12-min preincubation at 56°C, that of control rats was much affected by a preincubation period (Figure 2B).

These results showed that preincubation temperature and period were of no significance for the activation of kidney arginase in control rats.

Influence of pH on liver and kidney arginases

To determine optimum pH for arginase activity, various buffers were tested. These buffers were 100 mM Glycine-NaOH (pH 8.6–10.6), NaHCO_3 - Na_2CO_3

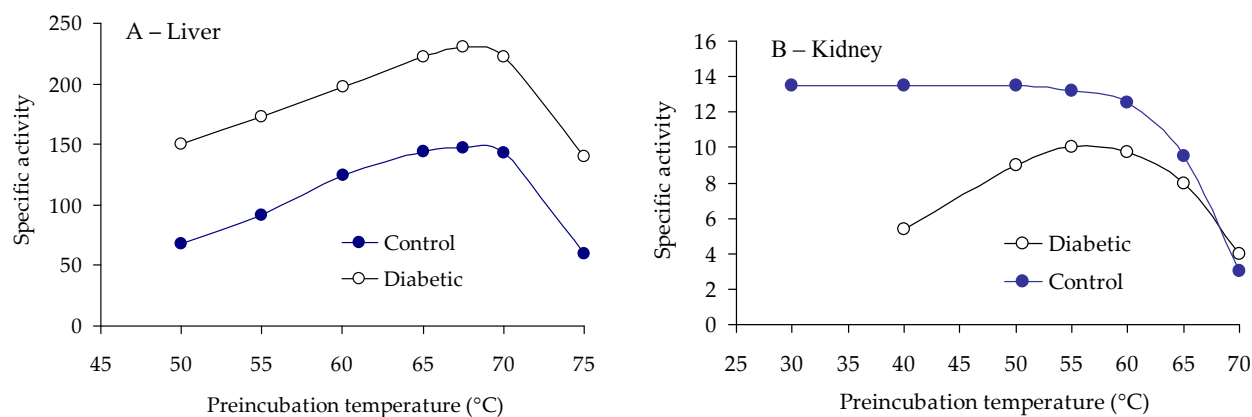


Figure 1. Influence of preincubation temperature on liver and kidney arginase

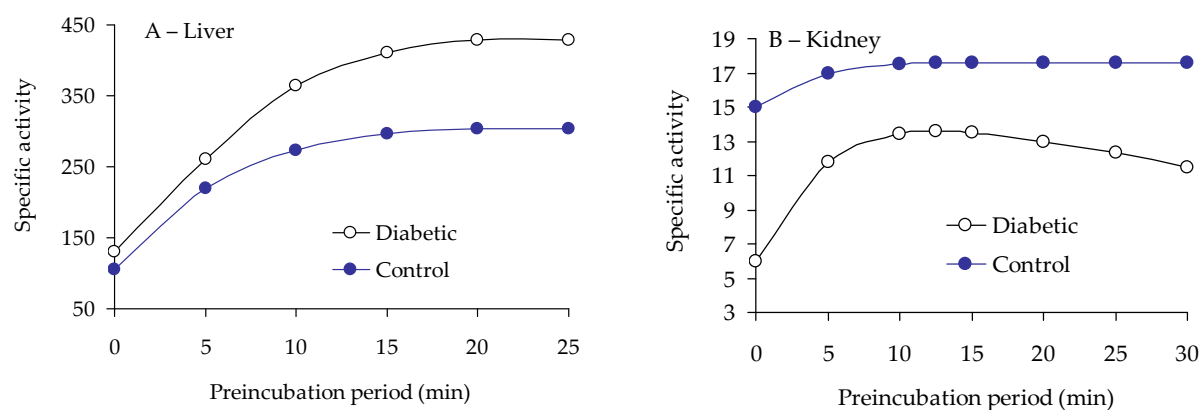


Figure 2. Influence of preincubation period on liver and kidney arginase

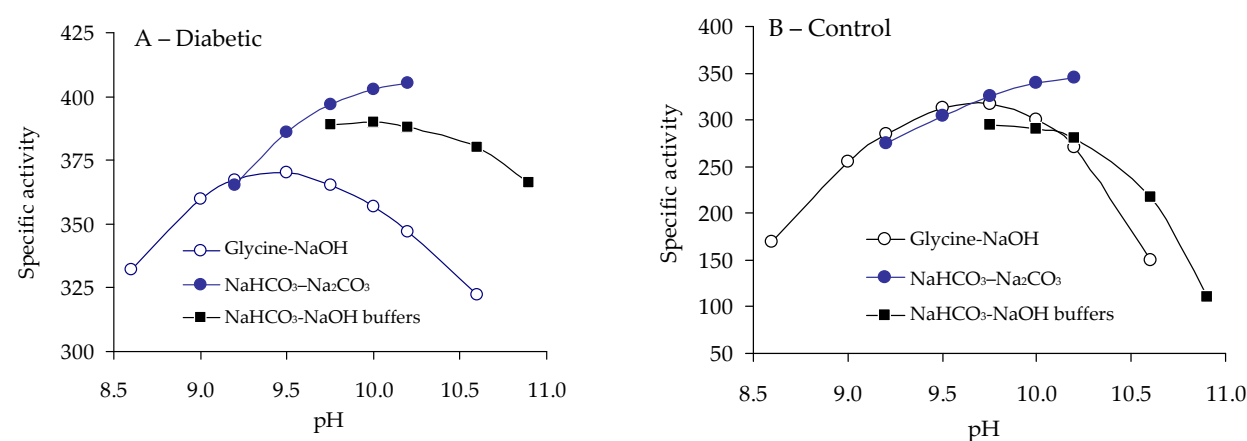


Figure 3. Relation between pH and catalytic activity of liver arginase

(pH 9.2–10.2), and NaHCO₃-NaOH (pH 9.7–10.9). The pH profile of arginase in liver of control and diabetic rats was similar. The arginase activity was highest at NaHCO₃-Na₂CO₃ buffer, pH 10.1–10.2 (Figure 3). The pH profile of arginase in kidney of diabetic rats

was different from that of control rats. In diabetic rats, with increasing pH, arginase activity in kidney increased and all the buffers showed virtually identical pH optimum curves with the maximum at 9.5 or above. We decided to use NaHCO₃-Na₂CO₃ buffer,

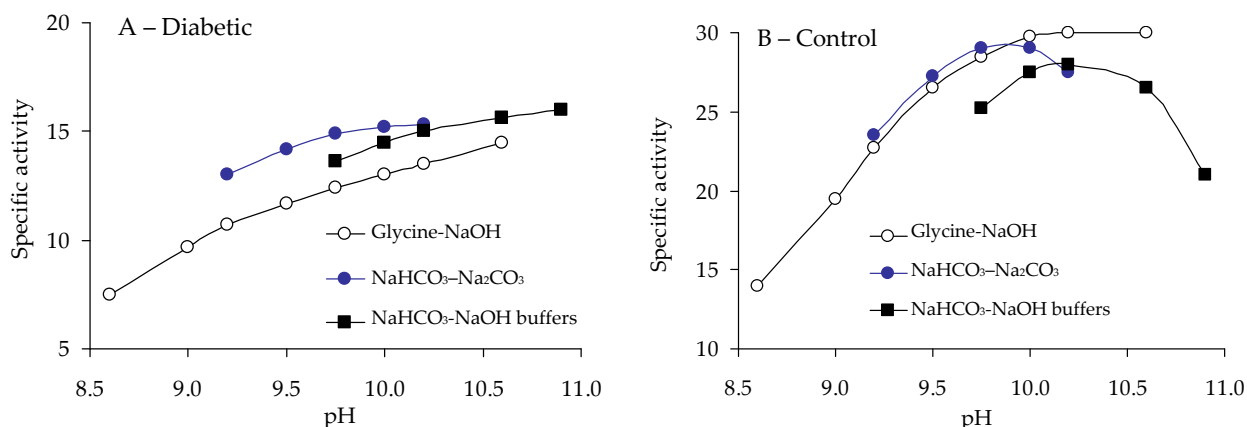


Figure 4. Relation between pH and catalytic activity of kidney arginase

pH 9.9–10 for the assay of kidney arginase activity in both control and diabetic rats (Figure 4).

Manganese dependence

To determine the influence of Mn²⁺ ions on arginase activity, Mn²⁺ ions at varying concentrations

were added during preincubation. While preincubation at 68°C for 20 min with an Mn²⁺-concentration of 2 mM fully activated liver arginase of diabetic rats, an Mn²⁺-concentration of 1 mM fully activated that of control rats. In the presence of 2 mM Mn²⁺-concentration in diabetic rats, the average activity after preincubation was higher 17 times than preincubation without Mn²⁺. In control rats, the presence of

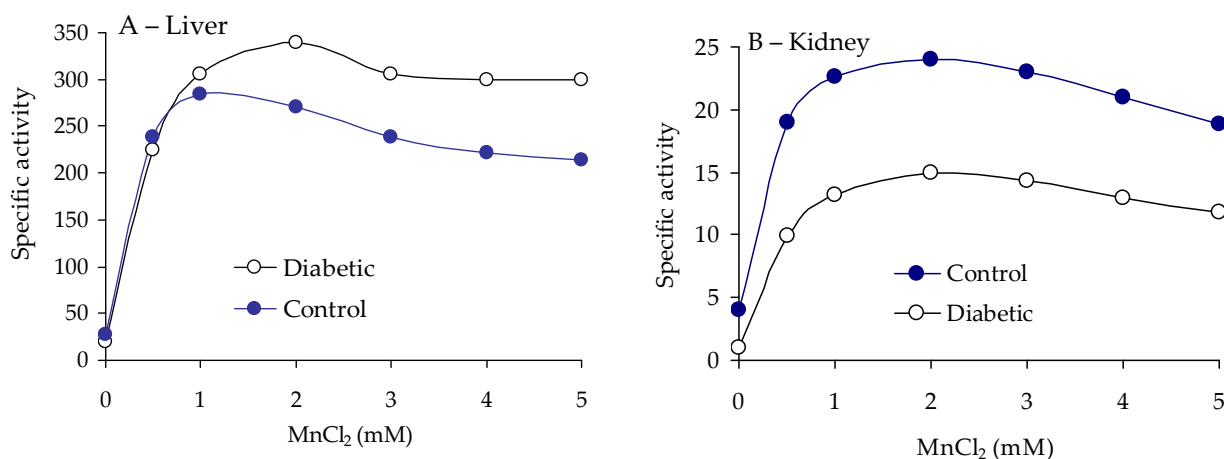


Figure 5. Effect of manganese concentration on arginase activity

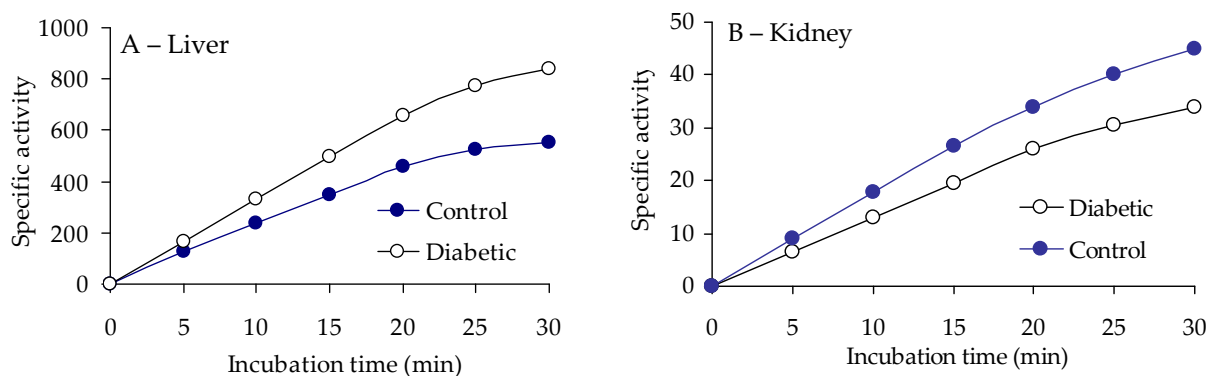


Figure 6. Influence of incubation period on arginase activity

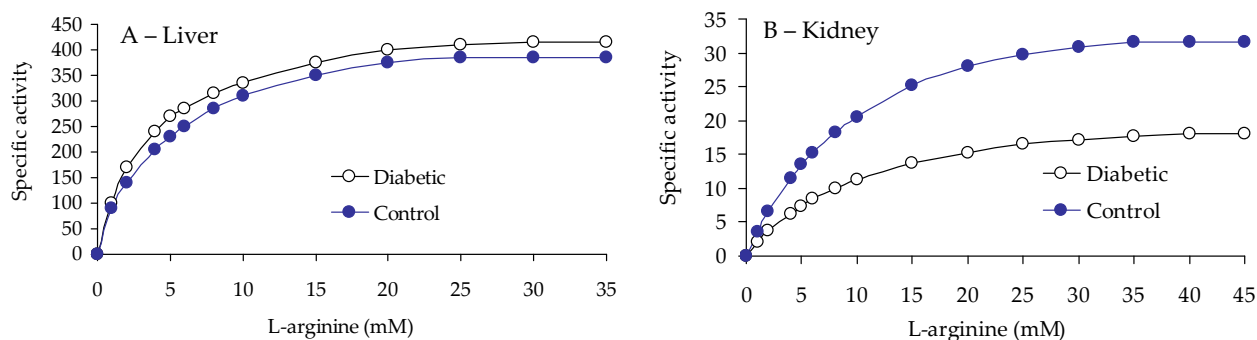


Figure 7. The changes in arginase activity at various L-arginine concentrations

1 mM Mn^{2+} -concentration increased the enzyme activity 10 times (Figure 5A). The highest arginase activity in kidney of both control and diabetic rats was detected at 2 mM Mn^{2+} -concentration. The Mn^{2+} -concentration of 2 mM activated kidney arginase 15 times in diabetic rats and 6 times in control rats (Figure 5B).

Influence of incubation period on liver and kidney arginases

In both control and diabetic groups, liver arginase activity is linear for 20 min at 37°C (Figure 6A). In contrast to the control (20 min), kidney arginase activity of diabetic rats was linear for less than 15 min (Figure 6B).

Michaelis-Menten constant

The K_m and V_{max} values of arginase were assessed according to Michaelis-Menten kinetic analysis. The K_m and V_{max} values of arginase for L-arginine in liver are very similar in control and diabetic groups. The K_m and V_{max} were 3.16 ± 0.18 and 379.47 ± 23.26 , respectively (Figure 7A). The K_m values (6.69 ± 0.12 mM) of arginase for L-arginine

in kidney of both control and diabetic groups were very similar. The V_{max} values for kidney arginase of diabetic group were lower than those of control group (Figure 7B).

Activities of liver and kidney arginases in diabetes

The activity of arginase in the liver of rat is about 15 times higher than in the kidney. The activity of arginase in the liver of diabetic animals was higher 1.5 to 1.7 times than that of controls (Table 1), whereas the arginase activity in the kidney of diabetic animals was found to decrease compared to controls. Diabetes caused an about 53% decrease in arginase activity in kidney of female rats, and 26% in that of males (Table 2).

DISCUSSION

It is well known that arginase activity is Mn^{2+} dependent. Mn^{2+} ions have been found to activate and/or stabilize arginases from different tissues (Kaysen and Strecker, 1973; Tarrab et al., 1974; Konarska and Tomaszewski, 1975; Van Elsen and Leroy, 1975; Spector et al., 1982; Scolnick et al.,

Table 1. Arginase activity (units/mg protein) in the liver of normal and diabetic rats

	Control group		Diabetic group		P
	n	Specific activity $\bar{x} \pm s_x$	n	Specific activity $\bar{x} \pm s_x$	
Male	8	347.78 ± 13.23	10	595.28 ± 22.86	< 0.001
Female	12	314.76 ± 6.55	8	485.21 ± 19.36	< 0.001
P		–		< 0.01	
Mean	20	327.97 ± 7.38	18	546.36 ± 19.95	< 0.001

Table 2. Arginase activity (units/mg protein) in the kidney of normal and diabetic rats

	Control group		Diabetic group		<i>P</i>
	<i>n</i>	Specific activity $\bar{x} \pm s_{\bar{x}}$	<i>n</i>	Specific activity $\bar{x} \pm s_{\bar{x}}$	
Male	8	23.53 \pm 2.40	10	17.38 \pm 0.61	< 0.05
Female	12	22.29 \pm 1.88	8	10.54 \pm 0.54	< 0.001
<i>P</i>		–		< 0.001	
Mean	20	22.78 \pm 1.45	18	14.34 \pm 0.91	< 0.001

1997). Liver and kidney arginases in both control and diabetic groups are also Mn^{2+} dependent (Figure 5). Likewise, diabetes changed the optimum Mn^{2+} concentration for rat liver arginase from 1 to 2 mM (Figure 5A). An increase in free Mn^{2+} concentration in the liver of streptozotocin-induced diabetic rats was established (Bond et al., 1983, 1986; Failla, 1986). Spolarics and Bond (1989) also determined that the K_m values for Mn^{2+} of the acidic forms of liver arginase from diabetic mice were higher than those of controls.

Arginase measured in various tissues required heat activation with Mn^{2+} before incubation in order to reach a maximum activity (Hirsch-Kolb et al., 1971; Glass and Knox, 1973; Spector et al., 1982; Erisir and Ozan, 1999). Previously, the optimum preincubation temperature for rat liver arginase was reported to be 60°C in the presence of $MnCl_2$ (Schimke, 1962; Hirsch-Kolb et al., 1971; Glass and Knox, 1973). Preincubation with Mn^{2+} at 68°C provided the maximum liver arginase activity in both control and diabetic groups (Figure 1A). Mouse and rat arginases are quite different with regard to thermal stability; the enzyme from diabetic and control mice is inactivated at 55°C in the presence of Mn^{2+} , for different periods of time (Spolarics and Bond, 1989). However, in contrast to the diabetic group (12 min at 56°C), the activity of kidney arginase in control rats was not increased by heat-treatment with Mn^{2+} (Figure 1B). Therefore the preincubation temperature and period for activation of kidney arginase in control rats was unnecessary. Furthermore, Kadowaki and Nesheim (1978) observed a decrease in the activity of the chick kidney enzyme prior to heat treatment (for 5 min at 55°C) with Mn^{2+} . These findings indicated that the arginase from rat liver in the presence of Mn^{2+} was a thermostable enzyme and much more resistant to elevated temperature than arginases

from other tissues such as kidney (Glass and Knox, 1973; Konarska and Tomaszewski, 1975; Kadowaki and Nesheim, 1978; Jenkinson and Grigor, 1994).

Mammalian arginases were previously shown to have basic pH optima (9.5–10.5). The optimum pH for the liver and kidney arginases in both control and diabetic groups is similar to that reported for other mammalian tissues (Tarrab et al., 1974; Konarska and Tomaszewski, 1975; Van Elsen and Leroy, 1975; Spector et al., 1982; Jenkinson and Grigor, 1994). However, the pH profile of arginase in kidney of diabetic rats was different from that of control rats.

The calculated K_m value in both control and diabetic groups was 3.16 ± 0.18 mM, similar to the value reported (3 mM) for rat liver enzyme (Tarrab et al., 1974). Jenkinson and Grigor (1994) reported that rat liver and kidney arginases gave the K_m values for the substrate, L-arginine, 14.2 and 14.4 mM, respectively. Kaysen and Strecker (1973) reported that the K_m value for kidney arginase was 18 mM, about two and a half times the K_m value for liver arginase (6.8 mM). On the contrary, the K_m value for rat liver arginase was found to be 1 ± 0.1 mM (Reczkowski and Ash, 1994). There were differences between the published K_m values for rat liver and kidney arginases (Glass and Knox, 1973; Kaysen and Strecker, 1973; Tarrab et al., 1974; Spector et al., 1982; Jenkinson and Grigor 1994; Reczkowski and Ash, 1994). This may be due to the variety of employed enzyme assays and to assay conditions that were not optimised.

Spolarics and Bond (1989) did not observe any change in the K_m and V_{max} values of mouse liver arginase as a consequence of diabetes. We also determined that the K_m and V_{max} values of rat liver arginase were unchanged in diabetes (Figure 7A). But there was an increase in the activity of arginase in liver of diabetic rats (Table 1). The activation

of liver arginase was observed in streptozotocin-induced diabetic rats and was shown to be due to an increase in free Mn^{2+} concentration in the liver (Bond et al., 1983, 1986; Failla, 1986). Mn^{2+} , a cofactor for arginase, is elevated in diabetic livers, and this divalent cation can modify the conformation (Schimke, 1962; Hirsch-Kolb et al., 1971; Scolnick et al., 1997) and the proteolytic susceptibility of the enzyme (Bond, 1973; Scolnick et al., 1997). On the other hand, an increase in mRNA concentration in the liver of diabetic rats was reported by Upadhyaya et al. (1999).

Kidney manganese content, by contrast, was similar in diabetic and control animals indicating that the altered manganese content of liver was tissue-specific (Bond et al., 1983; Failla, 1986). Similarly, we determined that the optimum Mn^{2+} -concentration required to obtain maximum catalytic activity in kidney of both control and diabetic rats was unchanged (Figure 5B).

The diabetic state is also associated with a great increase in urea excretion. Increased urea production in diabetes has been demonstrated *in vivo*, in liver slices, and in perfused livers (Green and Miller, 1960; McLean and Novello, 1965; Baxter and Schofield, 1980; Jorda et al., 1981). Both products (L-ornithine and urea) of the arginase reaction were inhibitors of arginase (Glass and Knox, 1973; Reczkowski and Ash, 1994). While K_m for kidney arginase is unchanged, a marked decrease in V_{max} may indicate enzyme inhibition (non-competitive) (Figure 7B). Our present data also indicated decreased kidney arginase activity under diabetic conditions (Table 2). We thought that the product inhibition and conformational changes of the enzyme might cause a decrease in kidney arginase activity in diabetes. On the contrary, an enhancement of the activity of arginase in diabetic kidney was noted by Upadhyaya et al. (1996). This may in part be due to assay conditions that were not optimised.

There are two distinct structural gene loci that encode arginase in the rat. The one structural gene locus expresses arginase in the liver, the other expresses arginase in the brain and kidney (Haggerty et al., 1983). We have confirmed that some physicochemical and kinetic properties of particularly kidney arginase of diabetic group are different from those of control group. These findings may support an idea that encoded arginases by separate gene loci may be differently affected by the pathological and hormonal status.

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Corresponding Author

Mine Erisir, Ph.D., Assistant Professor of Biochemistry, College of Veterinary Medicine, Firat (Euphrates) University, 23119-Elazig, Turkey
Tel. +90 424 237 6168, fax +90 424 238 8173, e-mail: mineerisir@yahoo.com
