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Immobilization, Characterization, and Laboratory-Scale Application of Bovine Liver Arginase

ERZSÉBET DALA AND B. SZAJÁNI*

Reanal Factory of Laboratory Chemicals
H-1441 Budapest 70, P.O.B. 54, Hungary

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ABSTRACT

Arginase isolated from beef liver was covalently attached to a polyacrylamide bead support bearing carboxylic groups activated by a water-soluble carbodiimide. The most favorable carbodiimide was *N*-cyclohexyl-*N'*-(methyl-2-*p*-nitrophenyl-2-oxoethyl) aminopropyl carbodiimide methyl bromide, but for practical purposes, *N*-cyclohexyl-*N'*-morpholinoethyl carbodiimide methyl tosylate was used. The optimal conditions for the coupling procedure were determined. The catalytic activity of the immobilized arginase was 290–340 U/g solid or 2.9–3.4 U/mL wet gel. The pH optimum for the catalytic activity was pH 9.5, the apparent temperature maximum was at 60°C and K_{mapp} was calculated to be 0.37*M* L-arginine. Immobilization markedly improved the conformational stability of arginase. At 60°C, the pH for maximal stability was found to be 8.0. The immobilized arginase was used for the production of L-ornithine and D-arginine.

Index Entries: Arginase, immobilized; support, polyacrylamide bead; carbodiimide, coupling agent; immobilized arginase, properties; L-ornithine, production; D-arginine, production.

INTRODUCTION

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) is the enzyme catalyzing the terminal step in urea production. It converts arginine into urea and ornithine. Arginase has been immobilized in different ways (*1–9*) and applied for practical purposes.

*Author to whom all correspondence and reprint requests should be addressed.

The arginine content of various mixtures can be determined by using an arginase-urease electrode. The arginine content of human serum and bovine insulin hydrolysate was assayed by Nikolelis and Hadjiioannou (*10*). Valle-Vega et al. (*11*) used the arginine content as a marker of the ripening of peanut. Rossi et al. (*12*) immobilized arginase isolated from bovine liver in the hollow fibers of a hemodialyzer.

Arginase is used for the enzymatic transformation of L-arginine to L-ornithine, which is applied in parenteral nutrition for liver disease therapy (*13,14*). For ornithine production, we prepared a covalently immobilized form of bovine liver arginase, using a polyacrylamide bead polymer as support. The immobilized enzyme was characterized as regards its catalytic properties and stability, and applied on a laboratory scale. The results are presented in this article.

MATERIALS AND METHODS

Arginase was isolated from bovine liver (*15*). Its specific activity was 34.6 U/mg protein. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the production of 1 μmol urea/min at pH 9.5 and 37°C. The Akrilex C-100 polyacrylamide bead support was a commercial product of Reanal Factory of Laboratory Chemicals (Budapest, Hungary) with a particle size of 100–320 μm and a binding capacity/COOH content of 5 meq/g solid. Carbodiimides were synthesized according to Jászay et al. (*16*). *N*-Cyclohexyl-*N'*-morpholinoethyl carbodiimide methyl tosylate was purchased from Fluka AG (Buchs, Switzerland). Folin phenol reagent was a product of Merck AG (Darmstadt, Germany). All other chemicals were reagent-grade commercial products of Reanal.

Immobilization of Arginase

Arginase was covalently attached to a polyacrylamide support possessing carboxylic functional groups activated by a water-soluble carbodiimide. The procedure was based on the method of Szajáni et al. (*17*).

One gram of Akrilex C-100 xerogel was suspended and swollen in 50 mL of 0.1*M* potassium phosphate buffer, pH 5.0–8.0. The water-soluble carbodiimide, in a stoichiometric quantity relative to the carboxylic functional groups located on the support, dissolved in 25 mL of cold (0°C) buffer, was added with continuous stirring and cooling in an ice bath. After stirring for 5 min, 25 mL of an arginase solution (40 mg/mL in buffer) were added to the suspension. The reaction time was 48 h, with two 6-h periods of agitation at 0–4°C.

The suspension was then filtered by suction through a G2 sintered glass filter. The immobilized arginase was washed successively three times with 0.1*M* potassium phosphate buffer (pH 7.0), three times with the same buffer containing 1.0*M* sodium chloride, and three times again

with the buffer. Finally, the immobilized enzyme was washed with a large volume of water to remove the buffer ions and was lyophilized.

Assay of Arginase Activity

The catalytic activity of arginase was determined by using a method proposed by Boehringer Mannheim GmbH (*18*). For the soluble enzyme, a mixture containing 0.2 mL of 0.2*M* L-arginine solution (pH 9.5), 0.2 mL of distilled water, and 0.1 mL of enzyme solution (10 mg arginase/mL/10 m*M* manganese maleate buffer, diluted 1:500 with redist. water) was incubated for 5–30 min, and the reaction was then stopped by the addition of 4.5 mL of 0.03*M* tungstic acid solution. After standing for 5 min at 20–25°C and centrifugation (2 min, 3000 rpm), the supernatant was treated with a 60 m*M* diacetylmonoxime/3.3 m*M* thiosemicarbazide reagent in boiling water for 20 min. The samples were then cooled to 20–25°C, and absorbances were measured at 546 nm. For the calculation, a urea standard (12.5 m*M*) was processed in the same manner.

In the immobilized arginase activity test, the enzyme samples were stirred in a reaction mixture of 0.2 mL of 0.2*M* L-arginase solution and 0.3 mL of 0.02 m*M* manganese maleate buffer for an appropriate period (5–30 min). The reaction was stopped by filtration (a few seconds), and tungstic acid solution was then added. The further procedure was the same as for the soluble enzyme.

Protein Measurements

Protein was determined according to the method of Lowry et al. (*19*) as modified by Schacterle and Pollack (*20*). The amount of immobilized protein was calculated as the difference between the amount of protein introduced into the reaction mixture and the amount of protein present in the filtrate and washing solution after immobilization.

Stability Tests and Soluble and Immobilized Arginases

The heat treatments were performed in 10 m*M* manganese maleate buffer, with a reaction mixture volume of 2 mL. After appropriate incubation periods, the samples were rapidly cooled in an ice bath, and the residual activities were assayed at 37°C.

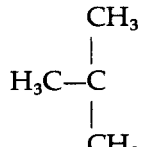
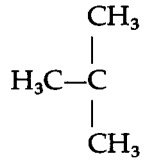
RESULTS

Immobilization of Arginase

Arginase was covalently attached to a polyacrylamide bead support possessing carboxylic functional groups activated by a water-soluble carbodiimide. The effects of the carbodiimide structure on the arginase immobilization were screened (Table 1). The most favorable carbodiimide structures for

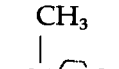
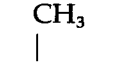
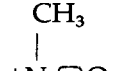
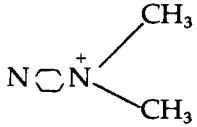
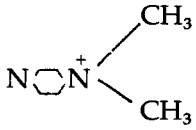
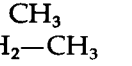
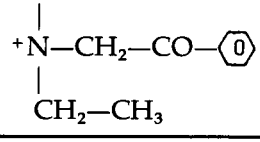
Table 1
Effect of Carbodiimide Structure on Immobilization of Arginase

Carbodiimide structure[a]					Activity on dry wt basis, U/g solid	Activity on protein basis, %[b]	Activity bound, %[c]	Activity loss, %[c]
R_1	R_2	R_3	n	x^-				
CH_3	CH_3	CH_3	3	J^-	120	1.83	4.2	91
CH_3	CH_3	CH_3	3	$H_3C-\langle O\rangle-SO_3^-$	141	0.82	3.7	60.8
CH_3	CH_3	$\langle O\rangle-CH_2$	3	Cl^-	110	1.1	0.2	55.8
CH_3	CH_3	$\langle O\rangle-CO-CH_2$	3	Br^-	270	0.7	7.3	56
CH_3	CH_3-CH_2	CH_3	3	J^-	27	0.9	0.1	52.9
CH_3-CH_2	CH_3	CH_3	2	J^-	250	2.6	9.7	82.5
CH_3-CH_2	CH_3	CH_3	3	Cl^-	330	58	4.3	15.7
CH_3-CH_2	CH_3	CH_3	3	$H_3C-\langle O\rangle-SO_3^-$	480	4.6	17.2	72.8
$CH_3-(CH_2)_2$	CH_3	CH_3	2	J^-	60	0.5	0.3	36.7
$CH_3-(CH_2)_2$	CH_3	CH_3	2	$H_3C-\langle O\rangle-SO_3^-$	180	3.1	0.9	54
$CH_3-(CH_2)_2$	$\langle O\rangle-CO-CH_2$	CH_3	2	Br^-	310	11	1.4	41.6
$CH_3-(CH_2)_2$	CH_3	CH_3	3	J^-	150	2.8	0.7	54.4
$CH_3-(CH_2)_2$	CH_3	CH_3	3	$H_3C-\langle O\rangle-SO_3^-$	113	1.3	0.5	85.5
$CH_3-(CH_2)_2$	CH_3	$\langle O\rangle-CO-CH_2$	3	Br^-	85	0.8	0.4	31.6
$CH_3-(CH_2)_3$	CH_3	CH_3	3	J^-	20	0.13	0.6	95.7
$CH_3-(CH_2)_3$	CH_3	CH_3	3	$H_3C-\langle O\rangle-SO_3^-$	330	2.72	10	83
$CH_3-(CH_2)_3$	CH_3	$\langle O\rangle-CO-CH_2$	3	Br^-	770	5.25	24	72.2

	CH_3	CH_3	3	J^-	610	6.5	20.3	68.7
	CH_3	CH_3	3	H_3C—⟨0⟩—SO_3^-	590	5.9	23	68.9
⟨0⟩	CH_3	CH_3	2	J^-	320	2	1.5	52
⟨0⟩	CH_3	CH_3	2	H_3C—⟨0⟩—SO_3^-	260	10.8	1.4	35.2
⟨0⟩	CH_3	CH_3	3	J^-	68	3	0.4	74.6
⟨0⟩	CH_3	CH_3	3	H_3C—⟨0⟩—SO_3^-	690	7.3	2.8	55.1
⟨0⟩	CH_3	⟨0⟩—CO—CH_2	3	Br^-	710	5.5	30.1	43.5
⟨0⟩	CH_3	O_2N—⟨0⟩—CO—CH_2	3	Br^-	1190	11.7	65.3	9.1

(*continued*)

Table 1 (Continued)

⬡			2	J^-	260	3.8	1.7	70
⬡			2	H_3C—⓪—SO_3^-	340	3.3	5.5	77
⬡			3	J^-	980	2.8	2.7	88.6
⬡			3	J^-	10	0.1	0.05	99.35
⬡			3	H_3C—⓪—SO_3^-	500	5	1.7	82.0
⓪		CH_3	3	J^-	550	13	2.2	70.8
⬡			3	Br^-	770	4.4	2.4	89.4

[a] General formula of carbodiimides, $R_1—N=C=N—(CH_2)_n—\overset{\overset{CH_3—}{|}}{\underset{\underset{R_2}{|}}{N^+}}—R_3X^-$

[b] The activity of the soluble enzyme was taken as 100%.

[c] The total activity introduced into the coupling reaction mixture was taken as 100%.

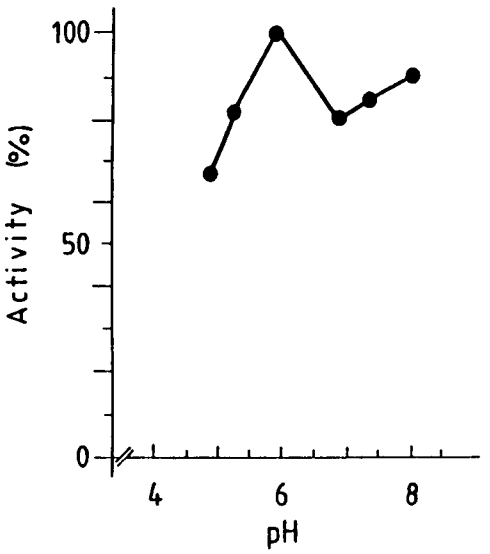


Fig. 1. Effect of pH of coupling reaction mixture on activity of immobilized arginase. Experiments were performed in 0.1*M* potassium phosphate at 0°C. The carboxylic functional groups of the support were activated with *N*-cycloexyl-*N'*-morpholinoethyl carbodiimide methyl tosylate. The maximum activity was taken as 100%.

high catalytic activity of the immobilized enzyme were those in which R_1 = cyclohexyl, R_2 = methyl, and R_3 = 2-*p*-nitrophenyl-2-oxoethyl, or R_2,R_3 = morpholinoethyl, n = 3, and X = bromide or iodide. In the further experiments, commercially available *N*-cyclohexyl-*N'*-morpholinoethyl carbodiimide methyl tosylate was used as coupling agent.

The most advantageous weight ratio of the support, carbodiimide, and protein was found to be 1:2:1. The highest catalytic activity was achieved when the coupling mixture was at pH 6.0 (Fig. 1). Under the optimum conditions, the highest activity achieved was 290–340 U/g solid or 2.9–3.4 U/mL wet gel.

pH Dependence of Catalytic Activity

The pH dependence of the initial rate of arginine conversion was studied at constant ionic strength in the pH range 8.0–12.0. After incubation of either the soluble or the immobilized enzyme, the pH was checked again, but no changes were found. The maximum rate (V_{max}) was determined at different pH values. The optimum pH for the catalytic activity of the immobilized enzyme was shifted in the acidic direction to pH 9.5, as compared with pH 11.0 for the soluble enzyme. Moreover, the shape of the pH profile was also altered (Fig. 2).

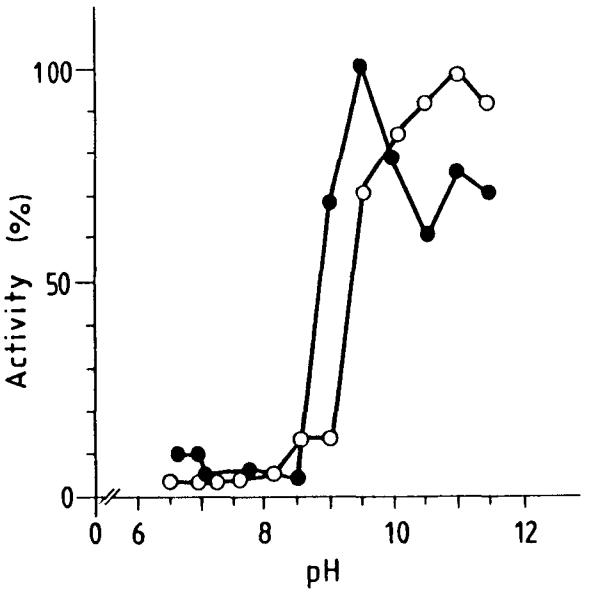


Fig. 2. Effects of pH on activities of soluble and immobilized arginase. Experiments were performed in 10 m*M* manganese maleate buffer at 37°C, with L-arginine as substrate. ○, Soluble enzyme; ●, immobilized enzyme. For both the soluble and the immobilized enzyme, the maximum activity was taken as 100%.

Temperature Dependence of Catalytic Activity

The temperature dependences of the activities of the soluble and immobilized arginase were studied in 10 m*M* manganese maleate buffer at the optimum pH for the catalytic activity (soluble enzyme, pH 11.0; immobilized enzyme, pH 9.5), with L-arginine as substrate, in the temperature range 25–70°C. Initial rates were found by measuring the activity for 10 min at selected temperatures. The apparent optimum temperature for the catalytic activity of the immobilized arginase was 60°C, i. e., much higher than that of the soluble enzyme (40°C) (Fig. 3).

Effect of Substrate Concentration on Catalytic Reaction

The effect of the L-arginine concentration on the initial rate of the catalyzed reaction was investigated in the concentration range from $2.5 \times 10^{-2}M$ to $5 \times 10^{-1}M$ at the optimum pH for catalytic activity (soluble enzyme, pH 11.0; immobilized enzyme, pH 9.5). Experiments were carried out in 10 m*M* manganese maleate buffer at the apparent optimum temperature for the catalytic activity (soluble enzyme, 40°C; immobilized enzyme, 60°C). K_m for the soluble enzyme was found to be $3.6 \times 10^{-2}M$, whereas K_{mapp} for the immobilized form was $3.7 \times 10^{-1}M$.

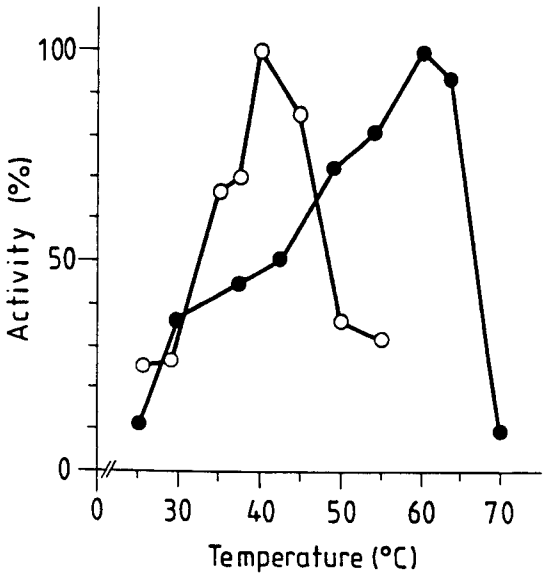


Fig. 3. Effects of temperature on activities of soluble and immobilized arginase. Experiments were performed at the optimum pH for the catalytic activity (soluble enzyme, pH 11.0; immobilized enzyme, pH 9.5) with L-arginine as substrate. ○, Soluble enzyme; ●, immobilized enzyme. For both the soluble and the immobilized enzyme, the maximum activity was taken as 100%.

Thermal Stability

The rates of thermal inactivation of the soluble and immobilized arginases were compared in 10 m*M* manganese maleate buffer at the optimum pH for the catalytic activity (soluble enzyme, 11.0; immobilized enzyme, pH 9.5) in the temperature range 40–70°C. At 40°C, the soluble enzyme was inactivated quickly, following first-order kinetics, with a $t_{1/2}$ value of 17 min. In contrast, at 40°C the immobilized enzyme exhibited very characteristic activation. At 60°C, the activation was only slight, followed by inactivation. The inactivation was very fast at 70°C (Fig. 4). The apparent half-life ($t_{1/2\ app}$) was calculated from the time curves: 34 min at 60°C and 3 min at 70°C. At 60 and 70°C, the soluble arginase practically lost its activity within 2 min.

pH Dependence of Thermal Inactivation/"pH Stability"

The effect of the hydrogen ion concentration on the thermal inactivation process was studied in 10 m*M* manganese maleate buffer in the pH range 6.5–9.5 at 60°C. Apparent half-lives ($t_{1/2\ app}$) derived from the time curves are presented in Fig. 5. The optimum pH for the stability of the immobilized arginase was pH 8.0, which is somewhat higher than that for the soluble enzyme (pH 7.5).

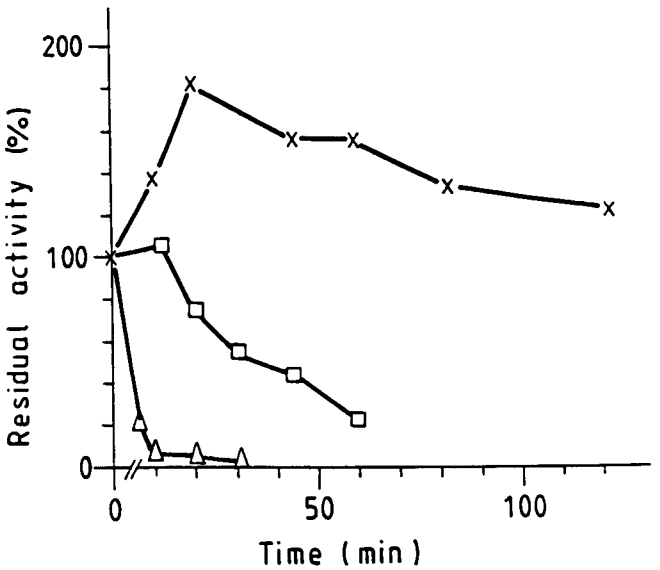


Fig. 4. Thermal inactivation of immobilized arginase. Experiments were performed in 10 m*M* manganese maleate buffer (pH 9.5). Enzyme concentration: 10 mg solid/mL. X, 40°C; □, 60°C; △, 70°C. The starting activity was taken as 100%.

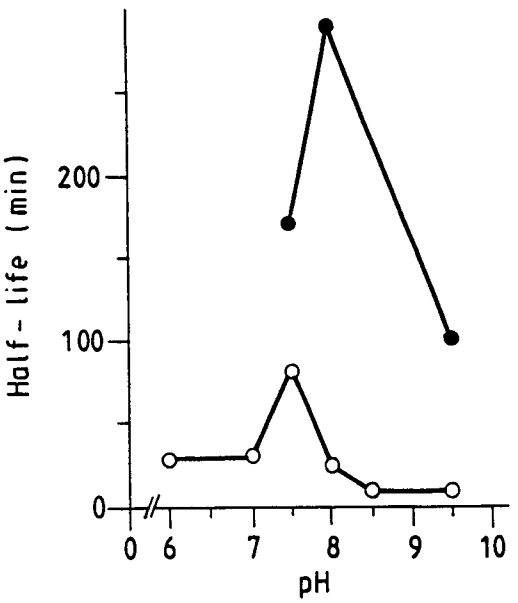


Fig. 5. pH-Dependence of thermal stabilities of soluble and immobilized arginase at 60°C. Experiments were performed in 10 m*M* manganese maleate buffer. Enzyme concentrations were 1 mg protein/mL (soluble enzyme) and 10 mg solid/mL (immobilized enzyme). ○, Soluble enzyme; ●, immobilized enzyme. The apparent half-lives ($t_{1/2\ app}$) were derived from the time curves.

Storage Stability

In lyophilized form, the immobilized arginase preserved its original catalytic activity for at least 5 mo at + 4°C.

Practical Use of Immobilized Arginase

The practical use of the immobilized arginase for the production of L-ornithine and the simultaneous production of L-ornithine and D-arginine from L-arginine and DL-arginine, respectively, was tested in preliminary experiments.

Production of L-Ornithine

Forty grams of L-arginine were dissolved in 1150 mL of distilled water, and the pH of the solution was adjusted to pH 9.5 with acetic acid. Then, 1 g of immobilized arginase xerogel (250 U) was added, and the mixture was stirred for 24 h at 25°C. Next, the enzyme was filtered off by suction, the pH of the solution was adjusted to pH 5.0 with hydrochloric acid, and it was evaporated to dryness *in vacuo* at 40°C. The solid was dissolved in 200 mL of toluene, and the solution was incubated for 12 h at 0–4°C. The L-ornithine·HCl was filtered off and washed with abs. ethanol. The yield was 31 g of L-ornithine·HCl (80.1% of theoretical).

Simultaneous Production of L-Ornithine and D-Arginine

Forty grams of DL-arginine were dissolved in 1150 mL of distilled water, and the pH of the solution was adjusted to pH 9.5 with acetic acid. Then, 1 g of immobilized arginase xerogel (250 U) was added, and the mixture was stirred for 24 h at 25°C. Next, the enzyme was filtered off by suction, the pH of the solution was adjusted to pH 11.0 with sodium hydroxide, and 57.4 mL of benzaldehyde were added at 5°C. The mixture was stirred for 4 h at 5°C. After incubation for 12 h at 0–4°C, the resulting benzylidene-L-arginine was filtered off and washed successively with ice water and diethyl ether at 0°C. The yield was 28.7 g of benzylidene-D-arginine (96% of theoretical).

The pH of the filtrate was adjusted to pH 1.0 with hydrochloric acid, and it was evaporated to dryness *in vacuo* at 40°C. The solid was then dissolved in 230 mL of abs. ethanol, and the solution was incubated for 1 h in an ice bath. After filtration, the pH of the solution was adjusted to 6.0 with triethanolamine. After incubation for 12 h at 0–4°C, the L-ornithine·HCl was filtered off and washed with abs. ethanol. The yield was 17.5 g of L-ornithine·HCl (90.2% of theoretical).

Twenty-five grams of benzylidene-D-arginine were incubated in a mixture of 40 mL of distilled water and 30 mL of concentrated hydrochloric acid at 60°C, with agitation. After cooling to 25°C, the mixture

was extracted three times with 30 mL of diethyl ether. The aqueous phase was evaporated to dryness *in vacuo* at 40°C. The resulting solid was dissolved in distilled water, the pH was adjusted to pH 6.0 with trietanolamine, and 100 mL of abs. ethanol were added. After incubation for 12 h at 0–4°C, the D-arginine was filtered off and washed with abs. ethanol. The yield was 19.3 g of D-arginine·HCl (95.5% of theoretical).

DISCUSSION

Arginase isolated from beef liver was covalently attached to a polyacrylamide bead support possessing carboxylic functional groups activated by a water-soluble carbodiimide. As regards the catalytic activity of the immobilized arginase, the most favorable water-soluble disubstituted carbodiimide was *N*-cyclohexyl-*N'*-(methyl-2-*p*-nitrophenyl-2-oxoethyl) aminopropyl carbodiimide methyl bromide, but for economic reasons, the commercially available *N*-cyclohexyl-*N'*-morpholino-ethyl carbodiimide methyl tosylate was used for practical purposes. The optimal conditions for the coupling procedures were determined. The most favorable composition of the coupling reaction mixture was a support: activating agent:protein ratio of 1:2:1 at pH 6.0. The catalytic activity of the immobilized arginase was 290–340 U/g solid or 2.9–3.4 U/mL wet gel. Surprisingly, in spite of the polyanionic microenvironment, the optimum pH for the catalytic activity of the immobilized enzyme was shifted in the acidic direction relative to that for the soluble enzyme. A similar phenomenon was experienced for immobilized cyclodextrin glycosyltransferase (*21*) and glucoamylase (*22*). The opposite direction of the shift relative to the theoretically probable direction indicates the importance of the specific interactions between the enzyme and the polymer matrix, such as the formation of covalent bonds between the functional groups of the matrix and certain amino acid side chains located on the surface of the enzyme molecules.

The apparent optimum temperature for the catalytic activity of the immobilized arginase was much higher than that for the soluble enzyme. It is suggested that the difference was caused by the enhanced conformational stability as a consequence of the strongly hydrophilic microenvironment. The direct effect of the microenvironment on the stability of the immobilized arginase was supported by the alkaline shift in the pH dependence of the thermal stability.

K_{mapp} of the immobilized arginase for L-arginine as substrate was an order of magnitude higher than that of the soluble enzyme, in spite of the opposite charge of the support and the substrate. The difference is presumably the result of complex interactions, including diffusion limitations.

The immobilized arginase could be used advantageously for the production of L-ornithine, and the simultaneous production of L-ornithine and D-arginine from L-arginine and DL-arginine, respectively.

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