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# Binding of Lichen Phenolics to Purified Secreted Arginase from the Lichen *Evernia prunastri*

**María-Estrella Legaz*, Carlos Vicente and Mercedes M. Pedrosa**
Laboratory of Plant Physiology, The Lichen Team, Faculty of Biology, Complutense University, 28040 Madrid, Spain

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**Secreted arginase from *Evernia prunastri* thallus has been purified 616-fold from the incubation medium. Purified arginase was resolved as only one peak in a capillary electrophoresis with a pI value of 5.35. The protein contained high amounts of acidic amino acids, such as Asx and Glx, and a relatively high quantity of Ser and Gly. The molecular mass of native, purified arginase was estimated as about 26 kDa by SE-HPLC. Substrate saturated kinetic showed a typical Michaelis-Menten relationship with a $K_m$ value of 3.3 mM L-arginine. Atranorin behaved as a mixed activator of the enzyme (apparent $K_m$ = 0.96 mM); whereas evernic and usnic acid were revealed as non competitive inhibitors (apparent $K_m$ values were 3.16 mM and 3.05 mM, respectively). Kinetics of atranorin binding indicated that saturation was reached from 0.18 µmol of the total atranorin and the occurrence of multiple sites for the ligand. This agrees with a possible aggregation of several enzyme subunits during the interaction process. A value of binding sites of about 12 was obtained. The binding of evernic acid was saturated from 23 nmol of total phenol. The number of binding sites was about 5. The loss of the binding ability of evernic acid could be interpreted as a single negative cooperatively. Usnic acid behaves in a similar way to evernic acid, although the binding saturation occurs at 0.14 µmoles of the ligand. This binding appears to be unspecific, and has 28 usnic acid binding sites to the protein.**

**Keywords:** Binding kinetics, *Evernia prunastri*, Phenols, Secreted arginase.

## Introduction

The Arginases, purified from several sources, seemed to be polymeric enzymes. The Arginase from *Neurospora crassa* has a molecular mass subunit of 38.3 kDa, as determined by SDS-PAGE (Borkovich and Weiss, 1987a). However, the polymer can be dissociated into two major immunoreactive proteins of molecular mass 41.7 kDa and 36.1 kDa, which are identified with polyclonal antibodies (Borkovich and Weiss, 1987b). The native, polymeric enzyme from *N. crassa* behaves as a hexamer with an apparent molecular mass of 266 kDa. On the other hand, mouse liver arginase is an oligomer composed of four subunits with a molecular weight of 35 kDa and 38 kDa, respectively (Spolarics and Bond, 1988).

*Evernia* arginase is a pool of different native proteins. Both induced and constitutive arginases have been characterized from *E. prunastri* thalli. The former has a molecular mass of 180 kDa (Legaz and Vicente, 1982), whereas the constitutive enzyme has a molecular mass of 330 kDa (Martín-Falquina and Legaz, 1984). Another form of arginase, with a molecular mass of 230 kDa, is secreted from the lichen thalli to liquid media (Planelles and Legaz, 1987). All of these isoforms are able to interact with some phenolic compounds that are produced by the lichen thallus. Part of these metabolites is retained inside the cells, whereas another part is secreted to the cortex. Thus, both the endocellular and exocellular forms of arginase could bind some of these phenols. In spite of their possible different genetic origin, the interconversion between induced forms of arginase of different molecular mass effected by phenols have been described (Legaz, 1991) in a similar way to that reported by Aguirre and Kashe (1983) for oligomeric rat liver arginase. Lichen phenolics are synthesized by the fungal partner of the lichen thallus, and used to regulate the size of algal population on the basis of their phycocidal activity (Honegger, 1987). They are alternatively partially secreted to the lichen cortex to produce a protective screen against UV radiation (Richardson, 1999). On the other hand, polyamine biosynthesis started by arginase activity is required for the maintenance of the vitality of algal populations (Molina and Vicente, 1995). Thus, regulation of secreted arginase by exocellular phenols is a point of great interest in the regulation of symbiotic equilibrium.

This paper describes the purification and some properties of

*To whom correspondence should be addressed.
Fax: 34-1-3945034
E-mail: melegaz@eucmax.sim.ucm.es

secreted arginase from *Evernia prunastri*, as well as the binding of some phenolics. They are produced by the same lichen species as the purified protein, which causes changes to its molecular mass.

## Material and Methods

**Plant material and incubation conditions** *Evernia prunastri* (L.) Ach., growing on *Quercus pyrenaica* Lam and collected in Valsaín (Segovia, Spain), was used throughout this work. Air-dried thalli were stored in polythene bags at 7°C in the dark until required, but no longer than two weeks. Samples of 50 g of air-dried thalli were floated on 1250 mL 40 mM L-arginine in 10 mM Tris-HCl, pH 9.15 at 26°C in the dark for 8 h.

**Purification procedure** Afterwards, the medium was filtered through Whatman N°3 filter paper, concentrated in air-flow up to 300 mL, filtered through Millipore GS filters (0.22 µm pore diameter), and immediately afterwards ammonium sulfate was added to reach a final concentration of 50% (w/v). The mixture was stored for 2 h at 4°C and then centrifuged for 1 h at 43,000 × g at 2°C. The precipitate was discarded, and the supernatant (containing the highest arginase activity) was dialyzed for 20 h against 5.0 L 10 mM Tris-HCl buffer, pH 9.1, at 4°C. Protein in the dialysate was then adsorbed on calcium phosphate gel, prepared according to Legget-Bailey (1967), and equilibrated with the same buffer, 75 mg dry gel *per* mg protein. Arginase was eluted from the gel with increasing concentrations of a Tris-HCl buffer, pH 9.1 (5 mM increments from 10 mM to 0.3 M). The eluate from 0.14 M contained the highest arginase activity. This eluate was electrofocused by using a 110 mL LKB 8100 column. The pH gradient was made with 1% (w/v) ampholines (Servalyt), pH 3.5-10, and the density gradient was prepared in 50% and 5% (w/v) sucrose solutions. The electrode solutions were 1.0 M NaOH at the catode, and 1.0 M $H_3PO_4$ at the anode The cooling temperature was 4°C, and the focusing was performed at 1000 V. Fractions of 2.0 mL were collected and assayed for arginase activity and pI value. The fraction that was eluted at pH 5.6, and contained the highest arginase activity (Pedrosa and Legaz, 1995), was used to assay its pI value by capillary electrophoresis.

**Capillary electrophoresis** Capillary zone electrophoresis was performed using the Spectraphoresis 500 system from Spectra-Physics (Fremont, USA), according to Legaz and Pedrosa (1993). Microbore fused silica tubing that was coated with polyamide (Scientific Glass Engineering, Kiln Farm Milton Keynes, United Kingdom) of 50 and 75 mm I.D., 190-360 mm O.D., respectively, was used with a total length of 70 cm, and a separation length of 63 cm. The capillary was enclosed on a cassette for easy handling. On-line detection was performed with a variable-wavelength UV-Vis absorbance detector with a 6 nm band width (Spectra-Physics). Detection of proteins was monitored at 200 nm and electropherograms were recorded using a SP 4290 integrator (Spectra-Physics).

Equilibration of the previously conditioned capillary was performed by washing it with different concentrations 15 mM or 25 mM of sodium borate-phosphoric acid buffer, pH 7.1 or pH 9.0, respectively, for 10 min at 25°C. After this, the capillary was washed again with the same buffer for 10 min at 25°C under 20 kV of applied voltage. This buffer system was chosen in order to produce a pH value higher than the isoelectric point (pI) of the proteins to be separated. This renders the proteins negatively charged, resulting in repulsion from the charged fused-silica capillary walls and thereby minimizing adsorption.

Tyroglobulin (pI = 4.5), alcohol dehydrogenase (pI = 5.4), carbonic anhyhdrase (pI = 5.9), and aldolase in A (pI = 6.6) or myoglobin in B (pI = 7.0), were purchased from Sigma Chemical Co. (St. Louis, MO, USA), kit No MW MWGF-1000. The neutral marker, benzol (pI equal to that of the electrolyte), was purchased from E. Merck (Darmstadt, Germany).

Protein solutions, prepared in the electrophoresis buffer and diluted three times, were injected into the capillary by syphoning for a fixed time of 8 s. Benzol at 4% (v/v) in the same diluted buffer was used as a neutral marker. Two different voltages, 17 kV and 25 kV, were applied using positive-to-negative polarity. During electrophoresis, temperature control was employed as indicated.

**Assay of enzyme activity** Arginase activity was assayed according to the Greenberg (1955) method, as modified by Legaz and Vicente (1982), which included crystalline urease in the reaction mixtures. These contained a final volume of 3.0 mL, 10 µmol Tris-HCl buffer, pH 9.1, 0.4 µmol L-arginine, 7.5 µmol maleic acid, 5.0 µmol manganese sulfate, 8.1 mg crystalline urease and 5.0 mg lichen protein. The reaction was stopped by adding 0.5 mL of a saturated potassium carbonate solution. The amount of ammonia produced was measured by the Conway (1962) microdiffusion method. Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. A unit of specific activity was defined as 1.0 µmol of ammonia produced *per* mg protein *per* min.

Kinetic data were determined by varying both substrate and effector concentrations in the reaction mixtures. Particular plots of these data were fitted by linear regression.

**Estimation of the molecular mass of secreted arginase** Molecular mass of purified, native arginase was estimated by SE-HPLC on a TSK-PW XL G5000 column (Legaz *et al.*, 1990a) that was equilibrated with 10 mM Tris-HCl buffer, pH 9.1. The void volume ($V_o$) was estimated from the elution volume ($V_e$) of standard blue dextran 2000. Molecular standards were tyroglobulin (669 kDa), apoferritin (440 kDa), β-amylase (200 kDa), ovalbumin (47 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.3 kDa) from Sigma Chemical Co. Protein in the eluate was monitored at 280 nm. The molecular mass of arginase was estimated by interpolating its elution parameter into a straight line made by plotting the log molecular mass *versus* log $V_e/V_o$ of the standards.

Alternatively, purified arginase was treated with 1% (w/v) sodium dodecyl sulfate and 2.5% (w/v) β-mercapto ethanol for 5 min in boiling water. Then the sample was filtered through an Alltech filter (0.45 µm pore diameter) to be loaded onto the chromatographic column. A SE-HPLC analysis was performed by using a Zorbax GF-450 column (25 cm × 9.4 mm I.D.) connected to a Zorbax GF-250 column (25 cm × 9.4 mm I.D.) from Dupont. Chromatographic conditions were as follows: loading, 10 µl;

mobile phase 0.2 M Tris-HCl buffer, pH 9.1, containing 1% (w/v) SDS; flux rate, 1.0 mL $min^{-1}$; pressure, 65 bars; temperature 22°C; detector, UV set at 280 nm.

**Amino acid analysis** Samples of 1.0 mL of purified arginase were dialyzed against distilled water to remove the salts from the buffer solution. Then, they were lyophilized and the residue dissolved in 0.2 mL 6 N HCl containing 0.1% (w/v) phenol and 20 nmol $mL^{-1}$ *nor*-leucine as an internal standard. Hydrolysis was carried out at 105°C for 24 h in sealed ampoules (Gavilanes *et al.*, 1982). An amino acid analysis was performed by using an amino acid analyzer, Beckman 6300, equipped with a module of analogic interface. Cys residues were analyzed as cystic acid after oxidation of the protein with performic acid (Elzinga and Hirs, 1968); whereas, Trp was spectrophotometrically quantified according to Beaven and Holiday (1952).

**Binding analysis of lichen phenols to secreted arginase** Binding of lichen phenols to purified secreted arginase was achieved by a equilibrium dialysis at 26°C for 30 min in metacrylate half-cells separated by a semipermeable membrane. One of two compartments contained 1.0 mL of 1.42 µM arginase. The other contained 1.0 mL of a phenol (atranorin, evernic acid or usnic acid) solution, which had a concentration that varied from 0 to 50 µM. A 1.0 mL Tris-HCl buffer substituted phenol solution was always performed in the controls. The number of moles of each ligand on the receptor, $L_p$ was calculated according to Klotz (1989), as $L_p = L_t - V_t/L$, where $L_t$ was the total moles of the ligand in the corresponding container, L, the moles of free ligand and $V_t$ the total volume of the solution, the sum of both compartments. After reaching equilibrium, each phenolic in both of the compartments was first extracted with diethylether : ethylacetate (65 : 35 v/v) and then analyzed by HPLC according to Legaz and Vicente (1983). The molecular mass of the protein after binding was estimated by SE-HPLC on a TSK column as above.

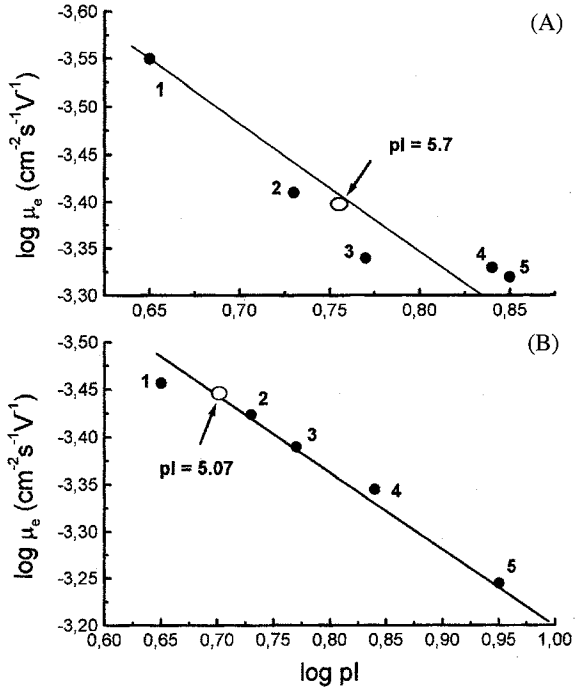


**Fig. 1.** Estimation of the pI value of secreted arginase from Evernia prunastri by capillary electrophoresis as a function of the electrophoretic mobility (($\mu_e$) by using 15 mM sodium borate/ phosphoric acid buffer, pH 7.1, at 30°C and 25 kV in (A), or a 25 mM sodium borate buffer, pH 9.0, at 30°C and 17 kV in (B). Regression equations for the straight lines were: y = -4.23 + 1.06x; r = 0.96, in (A) and y = -3.9 + 0.75x; r = 0.99, in (B). Markers were: 1 = tyroglobulin (pI = 4.5); 2 = alcohol dehydrogenase (pI = 5.4); 3 = carbonic anhydrase (pI = 5.9); 4 = aldolase (pI = 6.6) in (A) or myoglobin (pI = 7.0) in (B); and 5 = bencene. Empty circle represents secreted arginase.

## Results and Discussion

Secreted arginase was purified 616-fold (Table 1) from the incubation medium by conventional procedures. These include ammonium sulfate precipitation, adsorption chromatography and electrofocusing on liquid column with an overall yield of 5.6%. Purified arginase was resolved as only one peak in a capillary electrophoresis that eluted at 7.6 min by using as a electrolyte 15 mM sodium borate/phosphoric acid, pH 7.1, at 30° and 25 kV, or 10.5 min by using 25 mM sodium borate, pH 9.1, at 30°C and 17 kV. The pI of the

**Table 1.** Purification of secreted arginase from *Evernia prunastri* thallus floated for 8 h in the dark on 40 mM arginine in 10 mM Tris-HCl, pH 9.1.

| Fraction | Volume (mL) | Protein (mg $mL^{-1}$) | Total protein (mg) | Specific activity (units) | Total activity (units) | Recovery (%) | Purification (-fold) |
|---|---|---|---|---|---|---|---|
| Cell-free extract | 150 | 6.63 | 1990.20 | 0.20 | 398.04 | 100 | ----- |
| Supernatant from 50% ammonium sulfate precipitation | 75 | 0.22 | 16.57 | 20.90 | 346.25 | 86.98 | 104.45 |
| Fraction eluted with 0.14 M Tris-HCl, pH 9.1, from calcium phosphate gel | 45 | 0.03 | 1.35 | 68.89.50 | 93.00 | 23.36 | 344.45 |
| Fraction of pI = 5.6 from electrofocussing | 3 | 0.06 | 0.18 | 123.20 | 22.17 | 5.57 | 616.0 |

**Table 2.** Amino acid composition of secreted arginase from *Evernia prunastri*

| Amino acid | Mol (%) | Number of residues* |
|---|---|---|
| Cys** | 2.7 | 6 |
| Asx | 10.3 | 22 |
| Thr | 4.3 | 9 |
| Ser | 6.6 | 15 |
| Glx | 13.4 | 28 |
| Pro | 4.5 | 9 |
| Gly | 1.2 | 29 |
| Ala | 8.7 | 18 |
| Val | 6.6 | 14 |
| Met | 2.4 | 5 |
| Ile | 3.7 | 8 |
| Leu | 7.2 | 15 |
| Tyr | 2.8 | 6 |
| Phe | 3.1 | 7 |
| His | 2.1 | 5 |
| Lys | 5.3 | 11 |
| Arg | 4.7 | 10 |
| Trp*** | 9 | |

*Number of residues is expressed as the integer value next to the experimental one, assuming a molecular mass of the protein of 26 kDa.
**Determined as cysteic acid.
***Spectrophotometrically quantified.

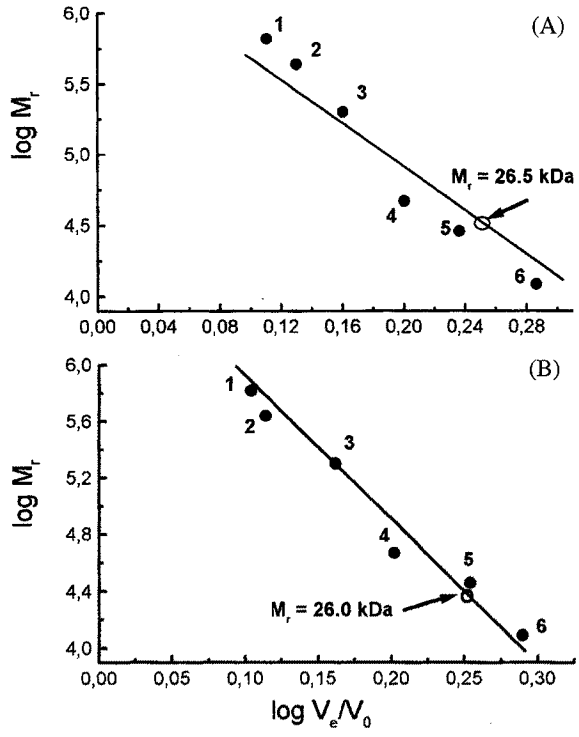


**Fig. 2.** Determination of the molecular mass of secreted arginase from *Evernia prunastri* by size-exclusion HPLC, using a TSK G5000 PWXL column in (A), where y = 6.49-8.65x; r = 0.98; and two Zorbax diol GF450-GF250 columns connected in series in (B), where y = 6.43-8.74x; r = 0.98. Molecular markers were: 1 = tyroglobulin (660 kDa); 2 = apoferritin (440 kDa); 3 = β-amylase (200 kDa); 4 = ovalbumin (47 kDa); 5 = carbonic anhydrase (29 kDa) and 6 = cytochrome c (12.4 kDa). Empty symbol represents secreted arginase.

protein was estimated as 5.7 (Fig. 1A) in the first conditions or 5.0 (Fig. 1B) in the second ones. The actual pI value of secreted arginase was then 5.7, as the nearest of that previously obtained by electrofocusing on a column (Pedrosa and Legaz, 1995). The amino acid composition of purified, secreted arginase is given in Table 2. The protein contained high amounts of acidic amino acids, such as Asx and Glx, and a relatively high quantity of Ser and Gly. The number of Trp residues was estimated as 9. The molecular mass of native, purified arginase was estimated as about 26 kDa by SE-HPLC on a TSK column (Fig. 2A). The same value of molecular mass was obtained after denaturation of the protein with SDS, and analysis by SE-HPLC on Zorbax columns (Fig. 2B). These results indicated that secreted arginase, purified according to that described in Methods, is composed of only one subunit.

Fluorescence emission spectra of purified arginase were recorded by exciting the peptide with light of 257, 275 and 295 nm. Only one peak of fluorescence emission at 349 nm was shown by exciting the protein with light of 257 nm. This wavelength was used to reveal Phe. Free amino acid shows a maximum of emission at 282 nm (Campbell and Dwek, 1984). This indicated that Phe residues in the arginase molecule occurred in a hydrophobic domain; thus they could be highly protected against the aqueous environment. Simultaneous excitation of Tyr and Trp residues by light of 275 nm produced two peaks of fluorescence emission at 305 and 345 nm; whereas excitation of Trp by light of 295 nm displaced these maxima at 331 and 351 nm, in addition a subsidiary peak of fluorescence emission appeared at 397 nm. Since the maximum of fluorescence emission from free Trp was recorded at 350 nm (Campbell and Dwek, 1984), the response of the protein indicated that Trp residues occurred in a very accessible domain in the protein, similar to that described for bovine serum albumin (Pico and Houssier, 1989). A main maximum of fluorescence emission at 355 nm (Trp) and a secondary maximum at 305 nm (Tyr), was shown by the simultaneous scanning of both excitation and fluorescence emission. (Fig. 3).

Substrate saturated kinetic showed a typical Michaelis-Menten relationship (Fig. 4) with a $K_m$ value of 3.0 mM L-arginine from a double-reciprocal plot (inset in Fig. 4). Secreted arginase seems to have a lower affinity by its substrate, arginine, than that shown for non-secreted, induced ($K_m$ = 2.0 mM), and constitutive ($K_m$ = 1.44 mM) arginases (Legaz *et al.* 1990a). Atranorin behaved as a mixed activator of the enzyme (apparent $K_m$ = 0.96 mM); whereas evernic and usnic acid were revealed as non-competitive inhibitors

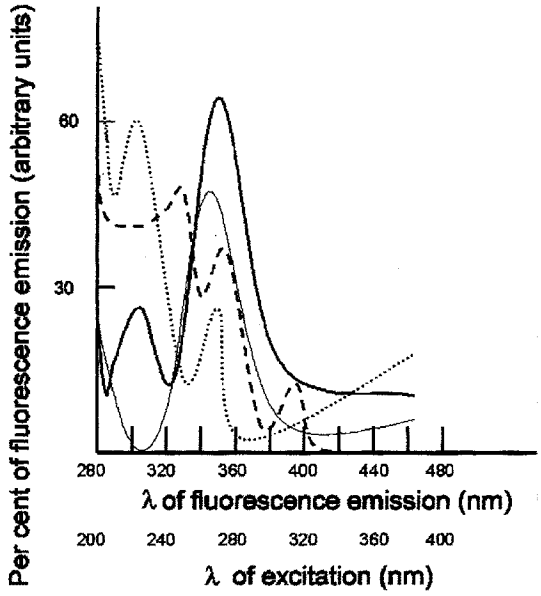


**Fig. 3.** Fluorescence emission spectra of secreted arginase excited with light of $\lambda = 257$ nm (fine line); $\lambda = 275$ nm (dotted line); $\lambda = 295$ nm (dashed line) or variable 1 of excitation (black line), as it is shown in the second X axis, with simultaneous recording of emission (spectrascan).

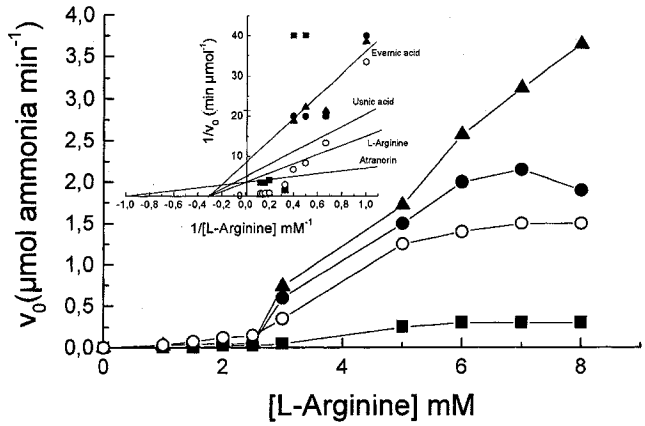


**Fig. 4.** Saturation kinetics of secreted arginase in the absence of any phenol (●) or in the presence of 0.19 μM atranorin (▲), 0.39 μM evernic acid (■), or 0.47 M usnic acid (❑).

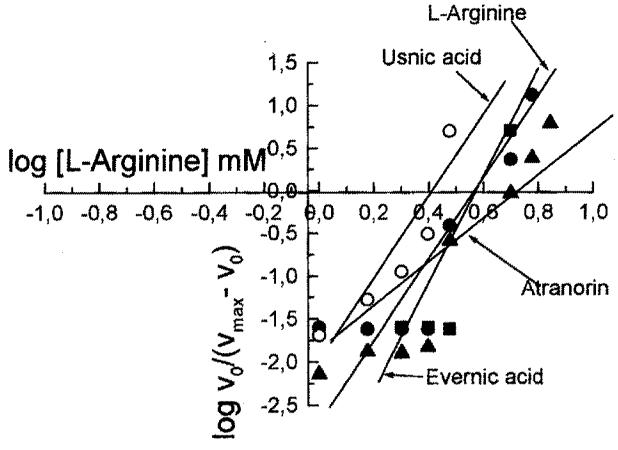


**Fig. 5.** Hill plot of the variation of the reaction rate of secreted arghinase as a function of the substrate concentration in the absence of any phenol (●) or in the presence of 0.19 μM atranorin (▲), 0.39 μM evernic acid (■), or 0.47 M usnic acid (❑).

(apparent $K_m$ = values were 3.16 mM and 3.05 mM, respectively). However, both atranorin and evernic acid behave as activators of a non-secreted, induced arginase; whereas evernic acid is shown to be an activator of the constitutive enzyme (Legaz *et al.*,1990b). The interaction coefficient (determined by Hill's plot) appeared to be $n_H = 2$ in the absence of an effector, as deduced from the slope of the corresponding straight line. It was somewhere near 1 in the presence of atranorin, but no significant changes were observed in the presence of evernic or usnic acids (Fig. 5).

Studies on the binding of these phenolics to the protein were performed. Fig. 6A shows the saturation kinetic of binding ($L_p$) as a function of the concentration of total atranorin ($L_t$). The curve obtained indicated that saturation was reached from 0.18 μmol of total atranorin and the occurrence of multiple sites for the ligand; this agreed with a possible aggregation of several enzyme subunits during the interaction process. The inverse of free ligand concentration *versus* fractional saturation of the binding sites ($r = L_p$/protein) clearly showed an exponential curve (Fig. 6B). The intercept of this curve on Y axis gave 23 sites for atranorin binding, indicating a high unspecificity of the binding. A Scatchard plot indicated that the highest value of $r/[L]$ was reached for $r$ values near 12 (Fig. 6C). From this point, this binding behavior reverses in order to obtain values near the minimum observed for the lowest concentration of the total effector used here. A value of binding sites of about 16 was obtained from the semilogaritmic plot, $r$ versus log $[L]$ (Fig. 6D). Since discrepancies of binding data that came from the different plots are insignificant when the number of binding sites is higher than 3, the most accurate value must be that obtained from the Scatchard plot according to Neet and Ainslie (1980)

It is still unknown whether or not hysteretic transition occurs after atranorin binding (Fig. 6C) together with changes in the quaternary structure of the enzyme. The Hill plot of binding appears to be the most appropriate for the quantitative assessment of positive cooperativity (Fig. 6D). A mean value near 2.0 for the Hill coefficient assures the influence of the first phenol molecule bound to the enzyme structure upon the succeeding ligand molecules in the process of arginase activation.

Fig. 7A shows the saturation kinetic of the binding of evernic acid to the protein. The binding saturated from 23 nmol total evernic acid. A exponential double-reciprocal plot (Fig. 7B) revealed that the number of binding sites was about 5. When these results were replotted according to a Scatchard plot, the higher the value of $r$, the higher the value of $r/[L]$ will be from the $r$ values from 0 to 3.8 (Fig. 7C). A

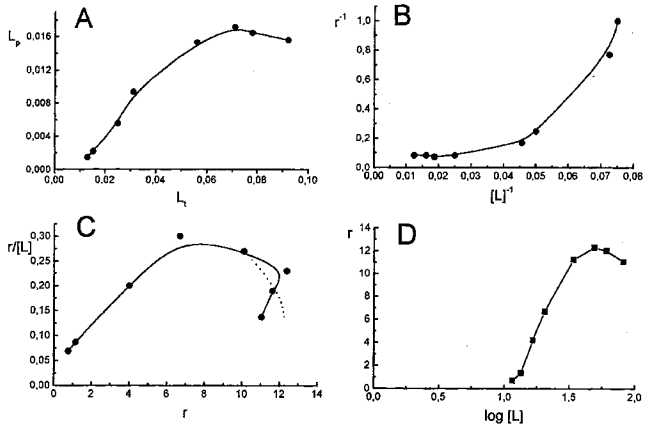


**Fig. 6.** Binding kinetics of atranorin to secreted arginase (1.42 μM). In (A) direct plot, where $L_p$ are mol of atranorin bound to the protein; $L_t$, mol of total atranorin; r, $L_p$ per mol of protein, and [L], concentration of free atranorin in the equilibrium. In (B) double-reciprocal plot in which the equation of the exponential function is $y = 0.04\ e^{39.38x}$; $r = 0.96$. In (C) Scatchard plot and in (D) semilogarithmic plot.

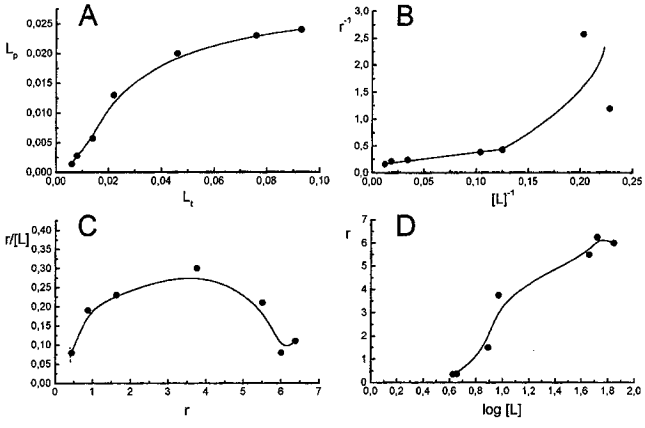


**Fig. 7.** Binding kinetics of evernic acid to secreted arginase (1.42 μM). In (A) direct plot, where $L_p$ are mol of evernic acid bound to the protein; $L_t$, mol of total evernic acid; r, $L_p$ per mol of protein, and [L], concentration of free evernic acid in the equilibrium. In (B) double-reciprocal plot in which the equation of the exponential function is $y = 0.13\ e^{11.44x}$; $r = 0.96$. In (C) Scatchard plot and in (D) semilogarithmic plot.

semilogarithmic plot was made to re-establish the number of binding sites in the protein (Fig. 7D), but the value obtained did not significantly change from that already obtained.

The loss of binding ability of the evernic acid for *r* values higher than 3.8 could be interpreted as a single negative cooperativity, since convexity of the direct plot is the expected result (Neet, 1980; Cera *et al.*, 1988). Thus, this second phase of binding kinetics can be defined as an additional state of the enzyme, which has different kinetical properties than those which define positive cooperativity (Ricard *et al.*, 1974; Philo *et al.*, 1988). The progress to the equilibrium curve for the high ligand concentration can be likened to that obtained for

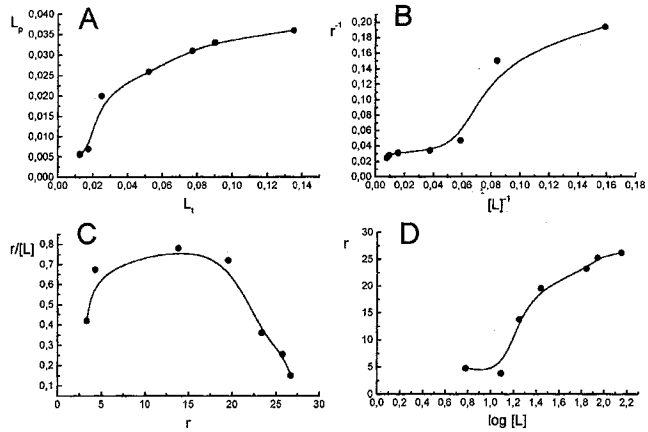


**Fig. 8.** Binding kinetics of usnic acid to secreted arginase (1.42 μM). In (A) direct plot, where $L_p$ are mol of usnic acid bound to the protein; $L_t$, mol of total usnic acid; r, $L_p$ per mol of protein, and [L], concentration of free usnic acid in the equilibrium.. In (B) double-reciprocal plot in which the equation of the exponential function is $y = 0.04\ e^{13.11x}$; $r = 0.85$. In (C) Scatchard plot and in (D) semilogarithmic plot.

other enzymes, such as phosphorylase (Metzger *et al.*, 1968) or hexokinase (Aisnlie *et al.*, 1972, 1975); there the enzyme, at very low concentrations, reacts with saturating amounts of the respective ligand. Since transition to this second stage is presumably accompanied by the inhibition of arginase activity, the ligand can shift the enzyme towards the second, lower active state rather than to accelerate the rate of transition to the inactive form (Neet and Ainslie, 1980).

Fig. 8A shows the saturation kinetic of the binding of the usnic acid to the protein. The binding is saturated from 0.14 mmol total evernic acid. An exponential double-reciprocal plot (Fig. 8B) revealed from the inverse of the intercept of the curve with the Y axis that the number of binding sites was about 26. This implied that the binding of usnic acid to secreted arginase was highly unspecific. When these results were replotted according to a Scatchard plot, experimental values were conveniently fitted to a parabole (Fig. 8C), similar to that described for evernic acid (Fig. 7C). A semilogarithmic plot was made to reestablish the number of binding sites in the protein (Fig. 8D).The value obtained, 28 sites, was very close to that obtained from the double reciprocal plot.

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