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PURIFICATION AND PROPERTIES OF THE ARGINASE FROM JERUSALEM ARTICHOKE TUBERS

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Abstract—Arginase [L-arginine amidinohydrolase] in Jerusalem artichoke tubers occurs in a particulate fraction from which it was released in active form by detergent treatment. The particulate enzyme was purified 450-fold with *ca* 3% yield. The enzyme has a MW of *ca* 140 000 and pI of 5.3. The enzyme required Mn^{2+} for activity and was unstable when Mn^{2+} was removed. In tissue extracts the K_m for arginine was *ca* 10 mM, but when purified the K_m (arginine) was 145 mM. The artichoke arginase was shown to be more substrate specific than other plant and animal arginases which have been described, and to be very sensitive to competitive inhibition by indospicine, ornithine and citrulline.

INTRODUCTION

The enzyme arginase, L-arginine amidinohydrolase (EC 3.5.3.1), occurs widely in nature. It has been studied extensively in animal tissues particularly in relation to nitrogen excretion and the Krebs–Henseleit urea cycle [1]. There are reports of arginase activity in a number of annual plants, mainly in seeds and seedlings [2–6] and it is found in leaves, fruit, seeds and seedlings of grapevines [7]. However, there is little knowledge of the intracellular location, the molecular structure or the kinetic properties of plant arginases.

In tuber tissues of Jerusalem artichoke (*Helianthus tuberosus*) free arginine forms a major portion of the nitrogen reserve. Arginine diminishes in tuber tissues when sprouting occurs in spring [8], when tuber tissue is sliced [9] and when callus growth is provoked [10]. Experiments with ^{14}C-labelled compounds implicate arginine as a proline precursor in this tissue, with ornithine as an intermediate [12]. Indospicine, an arginine analogue and an inhibitor of rat liver arginase [12], prevents both the loss of arginine and accumulation or proline which normally occur in osmotically stressed tuber slices [9]. The hydrolysis of arginine to ornithine by arginase is probably the initial step in this sequence.

In this paper, observations on arginase activity in artichoke tuber tissue through the autumn, winter and spring periods are reported. Methods for purifying the enzyme from both a total tissue extract and a particulate fraction are described and the molecular and kinetic properties of the artichoke enzyme are compared with those of arginases from other sources.

RESULTS

Seasonal changes in arginase activity

Enzyme activity in crude extracts of whole tubers was relatively low when the tubers were immature, but increased rapidly after growth of tubers ceased in May (Fig. 1). The highest activities encountered, 150–180 units/g fr. wt, were during winter dormancy and in early spring (late August) with the onset of sprouting. These activities are an order of magnitude greater than the arginase activity of grape tissues [7], but similar to that of *Vicia faba* cotyledons [6]. When the shoots were well developed in October and November, the levels of arginase activity in residual tuber tissue decreased. The specific activity of arginase was also highest around the sprouting time of the tubers (Fig. 1).

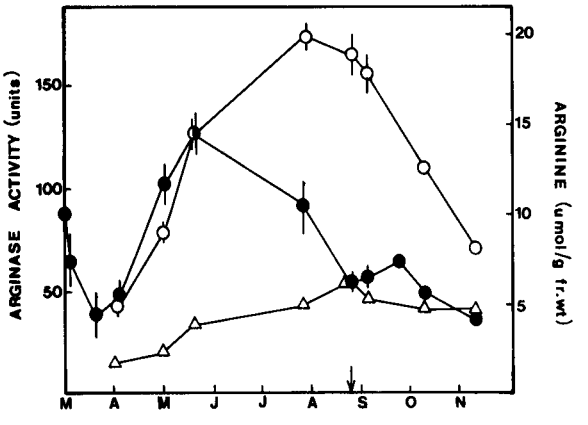

Fig. 1. Seasonal variations in the arginase and arginine content of field tubers. Total crude arginase activity in units/g fr. wt (○), arginine levels (●), and sp. act. of arginase in terms of units/mg total protein (△), are presented at various times of the year. The initial letters of the months beginning with March are shown on the abscissa, and the time at which sprouting began is indicated by the arrow. Each point is the mean of four analyses with s. d. indicated by the bars.

Free arginine in the tubers was maximal in April–May and low in late August when arginase activity was greatest (Fig. 1).

Location of arginase activity and purification

The arginase extracted from the whole tubers with TBD buffer (see Experimental) was purified *ca* 100-fold by fractional precipitation in acetone (50–66%), followed by column chromatography through Sepharose-6B and DEAE-cellulose. Acetone precipitation of whole tuber tissue homogenates resulted in an almost three-fold increase in activity, and thus an increase in specific activity. On the other hand, 70% of the activity was lost during DEAE-cellulose chromatography. Column isoelectric focusing of the DEAE-cellulose-treated enzyme resulted in a further 90% loss of activity. Both DEAE-cellulose chromatography and isoelectric focusing may remove Mn^{2+} from the enzyme.

Homogenates prepared using mitochondrial isolation medium were fractionated by differential centrifugation. A typical distribution of arginase activity is shown in Table 1; particulate arginase was 40–70% of the total at different times of the year. The bulk of the particulate activity was always distributed between the 0–2500-*g* and 2500–20 000-*g* pellets, the latter being rich in mitochondria.

The fraction sedimenting between 2500 and 20 000 (referred to as the 20 000-*g* pellet) was utilized for enzyme purification. The particulate enzyme was solubilized in 0.5% (v/v) Triton X-100 and then precipitated with acetone (Table 2). Further purification through DEAE-cellulose and Sepharose-6B resulted in a 450-fold purification and an overall yield of 2.6% (Table 2). Acetone treatment of enzyme released from particulate fractions caused no increase in activity, in contrast to the enzyme from whole tuber tissue extracts.

The purified, particulate arginase yielded one prominent and several minor bands on polyacrylamide gels. The major band had arginase activity. When eluted from the gel and examined by gel isoelectric focusing, the pure arginase zone yielded one protein band which corresponded with arginase activity.

Table 1. Subcellular distribution of arginase

Fraction	Total activity (units)	Sp. act. (units/mg protein)	Recovery of total activity (%)
Crude	2440	9.9	100
2500 *g* pellet	573	57.3	27.3
20 000 *g* pellet	880	84.6	41.9
80 000 *g* pellet	28	4.8	1.3
140 000 *g* pellet	18	2.0	0.9
140 000 *g* supernatant	602	3.8	28.7

Table 2. Purification of particulate arginase

Fraction	Activity (units)	Sp. act. (units/mg protein)	Recovery (%)
Homogenate	96 600	28	100
20 000 *g* pellet	28 920	204	29.9
DEAE-cellulose	7440	1860	7.7
Sepharose-6B	4280	4760	4.4
DEAE-cellulose	2520	12 600	2.6

Properties

The MW of the enzyme, isolated from whole tuber tissue, was 140 000 ± 20 000, as estimated from its elution pattern from a Sephadex G-200 column (75 × 2.6 cm) calibrated with alcohol dehydrogenase, bovine serum albumin, ovalbumin, myoglobin and cytochrome *c*. The MW of the crude enzyme released from the 20 000-*g* pellet, before and after gel isoelectric focusing, was estimated as 150 000 ± 10 000 from its behaviour on an Ultrogel AC 34 column (1.5 × 90 cm), calibrated with catalase, beef liver arginase and hexokinase. Column isoelectric focusing in a sucrose density gradient containing pH 3–10 ampholines concentrated the purified whole tuber tissue arginase in a fraction of pH 5.3. Gel isoelectric focusing of crude arginase released from the 20 000-*g* pellet resulted in several bands of activity with pI between 5.0 and 5.9.

Like most arginases, both crude and purified artichoke arginases were activated by Mn^{2+}. Maximum activity occurred with 1 mM Mn^{2+} in the assay medium. After removal of Mn^{2+} from the crude enzyme, for example, by ammonium sulphate precipitation, activity was partially restored by pre-incubation with 1 mM Mn^{2+} at pH 7.5. The restoration of activity was time and temperature dependent. Restoration of activity by preincubation was generally incomplete as found also with lupin arginase [13]. Crude artichoke arginase was stable at 55° for 30 min in the presence of 1 mM Mn^{2+}, but when Mn^{2+} was not added, 90% of the activity was lost after 15 min at 55°. The presence of Mn^{2+} was also essential during molecular sieve chromatography for maintenance of activity. An addition of Mn^{2+} to crude extracts also substantially increased activity at neutral and slightly acidic pH values, and shifted the pH optimum from 9.5 to 9.0 (Fig. 2).

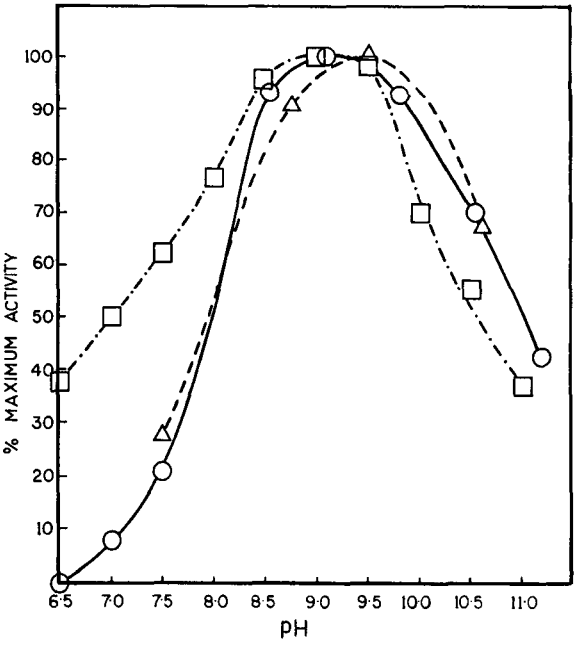

Fig. 2. Response of arginase to pH. The effects on crude whole tuber enzyme extracts assayed in the presence (□) and absence (△) of Mn^{2+} (extract B) are shown, together with the response of the purified enzyme (○) assayed in the presence of Mn^{2+}. The same result was obtained whether the enzyme was purified from the whole tuber extract or from the 20 000 *g* pellet.

Crude arginase in tissue extracts had a K_m (L-arginine) within the range 8–18 mM. After purification from either whole tissue extracts, or from the 20 000-*g* pellet (Table 2), the enzyme had a much-reduced affinity for substrate [K_m (L-arginine) 145 mM] and behaved in response to pH as though Mn^{2+} were absent (Fig. 2). Neither D-arginine nor canavanine were substrates for the enzyme in crude tissue extracts. At a concentration of 190 mM and pH 9.5, L-homoarginine was hydrolysed at 5% of the rate of L-arginine. The artichoke arginase is thus more substrate specific than are arginases from jack bean [2], lupin, ox liver or chicken liver [13–15].

A number of compounds chemically related to arginine were tested as inhibitors of arginase using equimolar concentrations of L-arginine and inhibitor (20 mM) and the unpurified whole tuber enzyme of low K_m. The observations are summarized in Table 3. None of these substances showed substrate activity and no evidence for a heteroarginase acting on γ-guanidinobutyrate [16] was found. In each case when a K_i value was determined (Table 3), inhibition was shown to be competitive. Indospicine (2-amino-6-amidino hexanoic acid) proved to be a potent inhibitor, and a kinetic analysis of this inhibition is shown in Fig. 3. Aspartate, glutamate, glutamine and proline were not inhibitory under these conditions. Artichoke arginase was inhibited by the thiol-binding reagent *p*-chloromercuribenzoate (PCMB) which at 18 μM caused an inhibition of 50%.

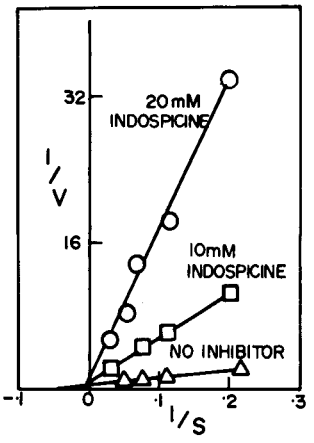

Fig. 3. Indospicine inhibition of arginase. *V* units are kat × 10^{-9}/50 μl enzyme. All points are the means of duplicates, with the error being no greater than 5%. Crude whole tuber extract after Sephadex treatment (extract A) was used for the kinetic studies.

DISCUSSION

The discovery in reptiles and birds of arginases with high MWs (*ca* 276 000) and low affinities towards arginine (K_m 100–200 mM) compared with those from mammals (MW 110 000–140 000; K_m 2–9 mM) led to the concept of 'uricotelic' and 'ureotelic' arginases [17]. In other words, arginases associated with animals which excrete nitrogen as uric acid were thought to differ from those found in animals which excrete nitrogen as urea. Later the correlation between arginase properties and type of nitrogen excretion was shown to be inexact [1]. A current division of arginases in animals is into two functional types, one acting preferentially on exogenous supplies of arginine and on arginine derived from protein hydrolysis, and the second linked with the urea cycle [18]. Arginases of the first type, catabolizing exogenous arginine, are unstable, particulate, neutral to slightly acid proteins. In contrast, the urea cycle enzymes are mostly stable, soluble, basic proteins.

Table 3. Inhibition of artichoke arginase

Inhibitor	Inhibition (%)	K_i (mM)
Indospicine	90	1–5
Canavanine	0	
Homoarginine	35	15
D-Arginine	16	
L-Citrulline	50*	1
L-Ornithine	50*	3
L-α-Aminobutyrate	32	10
γ-Aminobutyrate	0	
L-α-Aminoisobutyrate	50	10
Guanidinoacetate	33	
DL-α-Guanidinopropionate	49	
β-Guanidinopropionate	77	
γ-Guanidinobutyrate	65	

*Inhibitor concentration 4 mM; in all other cases inhibition was measured under standard conditions, with 20 mM L-arginine as substrate and 20 mM inhibitor. Crude whole tuber extract after Sephadex treatment (extract A) was used for these inhibitor studies.

The Jerusalem artichoke arginase is a Mn^{2+}-activated, acidic protein of MW 120 000–160 000. It becomes unstable during purification especially after methods such as ion-exchange chromatography, electrophoresis and isoelectric focusing, when Mn^{2+} may be removed from the protein. The artichoke arginase is predominantly particulate and the crude enzyme has a relatively low K_m. Its inhibition by PCMB distinguishes it from many of the liver enzymes, while it has properties in common with the non-urea cycle mammalian enzymes, e.g. rat kidney arginase [19]. These enzymes are believed to function in the catabolism of arginine to proline and glutamate via ornithine and it is these arginases which the Jerusalem artichoke enzyme most resembles. The similarity might also extend to intracellular location, as the arginases which are not associated with the urea cycle in mammals are predominantly particulate enzymes [19, 20]. *Vicia faba* arginase is also particulate [6]. Artichoke arginase is most likely a matrix rather than a membrane-bound enzyme, since it is solubilized entirely by TBD buffer (hypotonic) in the whole tuber extract.

Kinetic studies showed that the crude tuber arginase is not subject to feedback inhibition by urea, or by glutamate or proline, the products of ornithine catabolism. In contrast, various amino acids inhibit mammalian arginases [21]. Citrulline and ornithine are, however, powerful competitive inhibitors of the artichoke enzyme and inhibition by these metabolites may be important *in vivo*. If the main function for artichoke arginase is in the formation, ultimately, of proline and glutamate a critical role for ornithine in regulation *in vivo* is indicated by the observations that the K_i for ornithine inhibition of arginase, and the K_m for ornithine transamination (unpublished data) are each 3 mM. Thus if the reaction from arginine through to glutamic acid-γ-

semialdehyde is to proceed at a rapid rate, the ornithine formed by arginase must be sequestered away from that enzyme or 50% activity from both arginase and transaminase must be tolerable to the physiology of the plant. Muszynska *et al.* [13] found that ornithine inhibition of bitter lupin arginase was of the mixed-competitive type, a finding which contrasts with the present results for artichoke enzyme.

The instability of the purified arginase makes it difficult to interpret *in vivo* regulation in terms of observations on the enzyme in isolation. The large increase in K_m during isolation and the low yield during purification suggest that the purified enzyme may not be typical of the enzyme population in the cells. The strict Lineweaver–Burk kinetics of the crude enzyme suggest that there is only one form of arginase and that it undergoes major changes during isolation. In limited studies, active sub-units of the purified enzyme were not observed, but multiple forms of the crude enzyme were observed after gel isoelectric focusing. Either these forms are artifacts of the focusing method or they have very similar kinetic properties. Only one form remains after purification. It may be that the regulation of the native enzyme and its molecular architecture can be usefully studied only when methods of isolating enzyme of low K_m in high yield are achieved. The usefulness of arginine, Mn^{2+}, and to a lesser extent glycine, in protecting the enzyme from denaturation during early purification (e.g. molecular sieve chromatography) was noted during this study. Affinity methods based on arginine or metal binding, or utilizing the reversible competitive inhibitors available may be useful in arginase isolation.

EXPERIMENTAL

Tubers were grown and stored as previously described [9].

Extraction and preparation of crude enzyme extracts. Tubers were scrubbed, peeled and diced and then homogenized in a blender for 3 min with 1.5 vol. of a soln containing 50 mM Tris, 0.05 mM mercaptobenzothiazole, 5 mM dithiothreitol (DTT) and HCl to pH 8.2 (TBD buffer). The homogenate was filtered through two layers of muslin and centrifuged for 20 min at 40 000 *g*. The supernatant was passed through a column of Sephadex G-25 equilibrated and eluted with 5 mM Tris–HCl buffer (pH 7.5) containing 1% (w/v) sorbitol, 1 mM DTT and 1 mM $MnSO_4$ (TSDM buffer). The excluded fraction was used to study the kinetic properties of the unpurified enzyme (extract A). Extraction and gel filtration of the enzyme were at 0–4°.

Removal of Mn^{2+}. Tissue extract, prior to passage through Sephadex G-25 was adjusted to 90% of satn by careful addition of solid $(NH_4)_2SO_4$ while maintaining pH 8. After standing for 30 min, the sample was centrifuged for 20 min at 12 000 *g*. The deposit was dissolved in a soln containing 5 mM glycylglycine, 1% (w/v) sorbitol, 1 mM DTT and HCl to pH 7.8. The sample was passed through a column of Sephadex G-25 equilibrated with the same buffer, and the excluded eluate was used to study the properties of the Mn^{2+}-deprived enzyme (extract B).

Enzyme assay. Arginase activity was measured as the formation of urea in a medium containing 190 mM arginine and 1 mM $MnSO_4$ at pH 9.5. Arginine was normally a sufficient buffer, but when lower concns of arginine were used, 50 mM sodium glycinate, pH 9.5, was added. Enzyme was normally pre-incubated for 1 hr at 25° in 1 mM $MnSO_4$ at pH 7.5. Incubation with substrate was for 20 min at 25°, then 0.7 vol. of 87% HOAc was added and urea was measured in appropriate aliquots using a colorimetric method [22]. The assay was shown to be dependent on time and enzyme concn. Urea was verified as the product by the method of ref. [23]. No urease activity occurred during the assay. Citrulline interfered with the assay, and when citrulline was present, arginase activity was measured as the formation of ornithine [24]. Units of enzyme activity are expressed in kat $\times 10^{-9}$.

Analytical. Arginine and total and EtOH-soluble N were measured by methods described in ref. [9]. Protein was measured by the method of ref. [25] with bovine serum albumin as standard. To avoid interference by low MW substances, proteins were precipitated in 5% TCA and redissolved in 0.1 M NaOH before analysis.

Differential centrifugation. Tuber tissue was blended in 1 vol. of mitochondrial isolation medium as described in ref. [26]. The homogenate was subjected to the centrifugation series: 2500 *g* for 10 min; 20 000 *g* for 10 min; 80 000 *g* for 30 min; then 140 000 *g* for 90 min, with pellets being withdrawn at each stage. Enzyme was released from the pellets by incubation for 15 min at 25° in TSDM buffer containing 0.5% (v/v) Triton X-100 and centrifugation at 40 000 *g* for 20 min at 2°.

Sampling for seasonal changes. At appropriate times four lots each of six tubers were dug from the field, or taken from storage. The tubers were scrubbed and peeled, thus removing any roots or buds. The peeled tubers were split longitudinally. One half of each batch was finely chopped and a 20-g subsample was extrd in boiling 80% EtOH as described in [9]. The remaining half-tubers were homogenized in TBD buffer. This homogenate was subsampled for arginase, protein and total N estimates, using buffer blanks in each case. Substrate controls were included in the arginase assays. Enzyme and analytical measurements made on the matching batches of tuber halves agreed within 4%. When samples displaying high and low arginase activity were assayed together, the combined extract had the expected sum of the individual activities.

Arginase purification—particulate enzyme. Protein extrd with Triton X-100 treatment from the 20 000 *g* pellet obtained by differential centrifugation was precipitated first in 50% Me_2CO and then in 66% Me_2CO, both at −20°. The 50–66% ppt. was dissolved in TSDM buffer and added to a 29 × 1.4 cm column of DEAE-cellulose (Whatman DE52 microgranular) equilibrated with TSDM buffer without Mn^{2+} (TSD buffer). After washing with buffer a gradient of KCl from 0 to 1 M was applied in 600 ml of buffer at a flow rate of 25 ml/hr. DTT to a concn of 1 mM was added each 12 hr; 12-ml fractions were collected. Fractions containing arginase activity were mixed with 1 vol of Me_2CO, and the protein ppt. dissolved in TSDM buffer. This was fractionated on a 90 × 2.6 cm column of Sepharose-6B (Pharmacia) equilibrated with the same buffer. Elution was at 14 ml/hr.

The eluate was absorbed on a second DEAE-cellulose column (14 × 1 cm), washed in with 30 ml of TSD buffer and eluted by a 200-ml gradient of 0–0.3 M KCl.

The purity of the isolated enzyme was assessed using PAGE [27] and isoelectric focusing in vertical gels using pH 3–10 ampholines [28].

Purification of whole tuber extracts. The crude tissue homogenate prepared using TBD buffer was treated by fractionation with 50% and 66% Me_2CO, followed by a Sepharose-6B chromatography step similar to that used for the particulate enzyme. Active fractions were precipitated with an equal vol. of Me_2CO and subjected to DEAE-cellulose chromatography (TSD buffer, KCl 0–1 M). Bulked active fractions were again precipitated with an equal vol. of Me_2CO and the ppt. extrd with TSD buffer for column isoelectric focusing. A 110-ml column was used with pH 3–10 ampholines and a development time of 48 hr.

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