Pergamon

Bioorganic & Medicinal Chemistry, Vol. 2, No. 7, pp. 617-626, 1994

0968-0896/94 $7.00 + .00

0968-0896(94)00050-6

An Enzymatic Route to L-Ornithine from Arginine—Activation, Selectivity and Stabilization of L-Arginase

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Abstract—The non-proteinogenic amino acid L-ornithine (L-Orn) can be conveniently obtained by enzymatic hydrolysis of arginine (Arg) with arginase (EC 3.5.3.1). Arginase from calf liver (V_{max} = 459 μmol/(min•mg), K_m = 25.5 mM) is inhibited competitively by L-Orn (K_i = 480 mM). The enzyme was found to be completely enantioselective (E-value > 100) so that D,L-Arg can be split into D-Arg and L-Orn. Operational stability at 25 °C (deactivation rate constant $k_{deact} = 3.8 \times 10^{-3}$ h^{-1}; $\tau_{1/2}$ = 182 h) is sufficient for use in a continuous process but is significantly smaller than temperature stability ($k_{deact} = 4.1 \times 10^{-4}$ h^{-1}; $\tau_{1/2}$ = 1682 h); mechanical stress through stirring and unsteady Mn^{2+} supply owing to oxidation in the continuous process are believed to cause the difference. Addition of ascorbic acid stabilizes calf liver arginase at temperatures higher than 25 °C (at 60 °C, $\Delta\Delta G^{\ddagger}$ = 2.9 kJ/mol).

Introduction

Purpose and scope

As components of parenteral nutrition solutions and pharmaceutical intermediates, L-arginine (L-Arg) salts can be replaced by salts of L-ornithine (L-Orn) which have superior physical properties and lead to lower urea load in the body. L-Orn salts can be obtained either through fermentation processes[1,2] based on sugars or from chemical or enzymatic transformation of L-Arg. While fermentations need large volumes for economical operation and while chemical hydrolysis of L-Arg results in by-products from racemization (D-Arg) and side reactions (citrulline), L-Orn is accessible by enzymatic hydrolysis of L-Arg.[3-5] This way, in large-scale synthesis as well as in the mammalian body as part of the urea cycle (Figure 1), L-Orn is obtained by hydrolysis of its guanidino group with L-arginase (L-arginine amidinohydrolase, EC 3.5.3.1)(eq. 1):

(1)

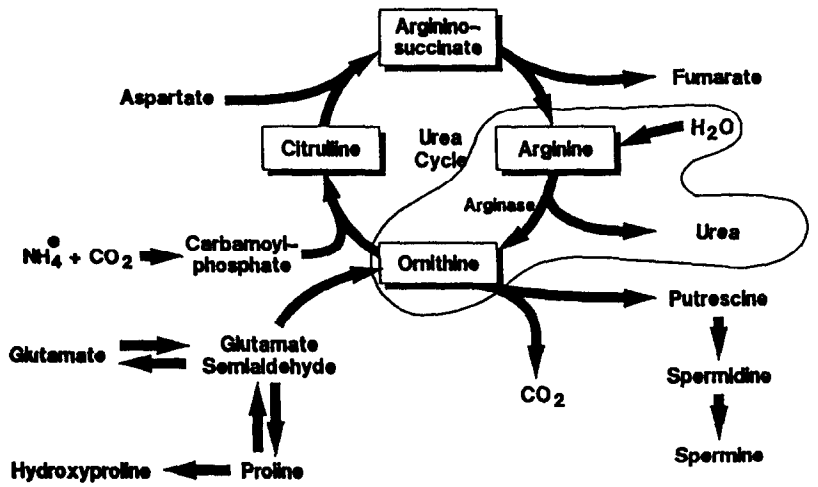


Figure 1. Arginase reaction in the urea cycle.

Abbreviations: D-Arg ((*R*)-Arg) = D-arginine, L-Arg ((*S*)-Arg) = L-arginine, L-Orn ((*S*)-Orn) = L-ornithine

One potential drawback of the enzymatic route is that arginase is available from commercial sources in diagnostic quantities only. The goal of this work was to check feasibility and potential of a large-scale synthesis of L-Orn salts based on commercially available arginase from calf liver.

Properties of calf liver arginase

Arginase is a metalloenzyme with narrow substrate specificity which can be isolated from different hepatic tissues (calf,[6] beef,[7] rat,[8] or human[9]), from other organs such as ox erythrocytes[10] but also from plants (jack beans[11] or iris bulbs[12]) or from microorganisms such as *Bacillus subtilis*[13] or *Saccharomyces cerevisiae*.[14] All mammalian liver arginases seem to be composed of four subunits with about 30 kDa each. Table 1 summarizes the properties of calf liver arginase.

Arginases contain four Mn atoms per protein molecule, which are essential for enzymatic activity and, in some cases, structural integrity of the protein molecule (for Mn affinity constants of calf liver enzyme, see Table 1). The structure of the active site is not clear, however, Cys does not seem to be involved.[15] Arginase is activated[16,17] and stabilized[4] by Mn^{2+} ions; at the optimum pH of 9.5–10,[17,18] 100 % activation is reached at 5×10^{-4} M;[18] for possible substitutions, see Table 1. The reason for activation of arginase by Mn^{2+} ions is reversible dissociation of some of the manganese with a concomitant loss of activity. After dialysis, the beef liver enzyme contains only one manganese per complete enzyme molecule and retains 25 % of its activity,[7] whereas addition of Mn^{2+} restores almost all of the original activity.

While calf and beef liver arginase have similar structure and properties (see Table 1), even other mammalian hepatic arginases differ significantly from calf liver arginase: rat liver arginase dissociates into subunits upon removal of manganese[19] (while calf liver enzyme does not) and has different dissociation constants of Mn^{2+} from calf liver enzyme [rat liver enzyme: two dialyzable Mn^{2+} ($K = 2 \times 10^4$ M^{-1}), two Mn^{2+} inaccessible to EDTA ($K > 3 \times 10^7$ M^{-1})].[20]

Preparation of D-arginine

There is an increasing demand for the unnatural amino acid D-Arg as part of pharmaceutical peptides such as LHRH antagonists.[21] Synthesis via resolution of a racemate of D,L-Arg derivatives is difficult: L-Arg is not a substrate for acylases[22] or amidases[23] and precursors cannot be synthesized by Strecker synthesis[24] because the corresponding aldehyde, 5-guanidinobutyraldehyde, is not

Table 1. Properties of calf and beef liver arginase

MW[19]:	120 kD (138 kD[16]; beef: 115 kD[7])	
Configuration[19]:	identical tetramer	
pI[6]:	4.05 (beef: 5.90[7])	
pH optimum[17,18]:	9.5-10	
metal atoms[19]:	4 Mn, 1 per subunit	
activator:	Mn^{2+} (optimum [Mn^{2+}] = 0.5 mM)[18]	
substitutions[18]: (rel. activity)	Cd^{2+} (32%; opt. pH 9.5-10)	
	Co^{2+} (23%; opt. pH 8.5-9)	
	Ni^{2+} (8%; opt. pH 8.5-9)	
	Mg^{2+}, Ca^{2+}, Cu^{2+}: inactive	
dissociation constants[19]: (o-phenanthroline)	$K_{1,2} = 2.0 \pm 0.5 \cdot 10^3$ M^{-1} (diss. at 4°C)	
	$K_3 = 4.2 \pm 1.7 \cdot 10^3$ M^{-1} (diss. at 37°C)	
	$K_4 = \geq 10^5$ M^{-1} (not diss. at 37°C)	
substrates[11,34]:	L-arginine	100%
	D-arginine	0%[11]
	L-canavanine	0.1%[11]
	L-homoarginine	2.0%[34]
	Nα-acetyl-arginine	3.9%[34]

available. However, owing to its basic nature, the abundant L-Arg can easily be racemized according to established procedures.[25] Based on the assumption, tested in this paper, that D-Arg is neither a substrate nor an inhibitor, arginase could be utilized for the co-synthesis of two valuable pharmaceutical intermediates:

$$\text{D,L-Arg} + H_2O \rightarrow \text{D-Arg} + \text{L-Orn} + \text{urea.} \qquad (2)$$

The applicability of eq. 2 depends on three conditions:

(i) calf liver arginase is sufficiently enantioselective; otherwise, the L-Orn product generated is not enantiomerically pure.
(ii) D-Arg is neither a strong nor a non-competitive inhibitor, so that the L-enantiomer of Arg is transformed completely, resulting in enantiomerically pure D-Arg.
(iii) D-Arg can be separated quantitatively from L-Orn.

D-Arg has been tested as a potential substrate; results were negative with arginase from mammalian livers (calf,[6] ox[26] and chicken[26]) but slightly positive with enzyme from plants (lupins[26] and jack beans[11]). However, the use of D,L-Arg as substrate has never been explored (although the idea has been voiced in ref.[3]) and neither has the effect of D-Arg as a potential inhibitor nor the enantioselectivity of any arginase. Separation of D-Arg from L-Orn might be achieved by ion exchange chromatography on a basic resin.

Results

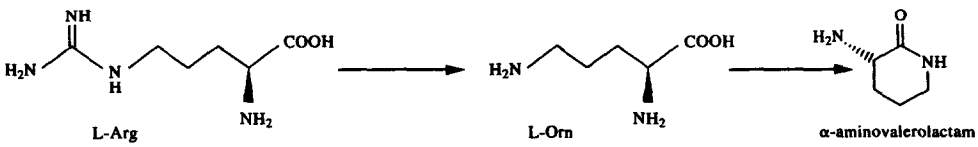
Activity of arginase

Activity vs pH profile. As was found earlier by our group[4] the activity vs pH profile of calf liver arginase extends far more to the alkaline side than reported previously (Figure 2).[16,17] Maximum activity was found in the pH range between 11.0 and 12.0. Some other arginases show similar properties such as arginase from ox erythrocytes[10] (pH optimum at 11.5). During most runs no buffer was added to the L-Arg substrate solution in order to keep a low salt level for simplified separation and work-up of the L-Orn product. Depending on initial concentration of L-Arg substrate, the initial pH is between 11.0 and 11.5, i.e. in the optimum pH range.

In the course of L-Arg hydrolysis to L-Orn, the pH decreases from around 11.5 to about 9.5. Even at pH 11.5, enzyme deactivation is slow enough not to cause significant damage. However, in this work we tested the hypothesis that arginase stability decreases with rising initial pH. As stated previously[4] conversion of L-Arg to L-Orn by arginase does not necessitate addition of a buffer.

Activation by Mn^{2+} ions and MnO_2 precipitation. In accordance with the literature[18] (however, see ref.[31]) an optimum concentration of the activator Mn^{2+} of 0.5 mM was found. At the prevalent alkaline pH values, MnO_2 from the oxidation of Mn^{2+} precipitates. To suppress oxidation, ascorbic acid was added as a reducing agent. It was found that ascorbate did not just prevent oxidation of Mn^{2+} at many conditions but also seemed to influence arginase activity decay (see below).

Kinetics. Figure 3 depicts conversion vs time profiles of the arginase-catalyzed conversion of L-Arg to L-Orn in a batch reactor; parameters are substrate concentration and L/D-ratio of Arg. In all cases, conversion to L-Orn is quantitative as expected from thermodynamics;[32] however, at high substrate concentration or long residence time, a small amount of α-aminovalerolactam can be detected. This results from a cyclization reaction of L-Orn product following the desired transformation:



(3)

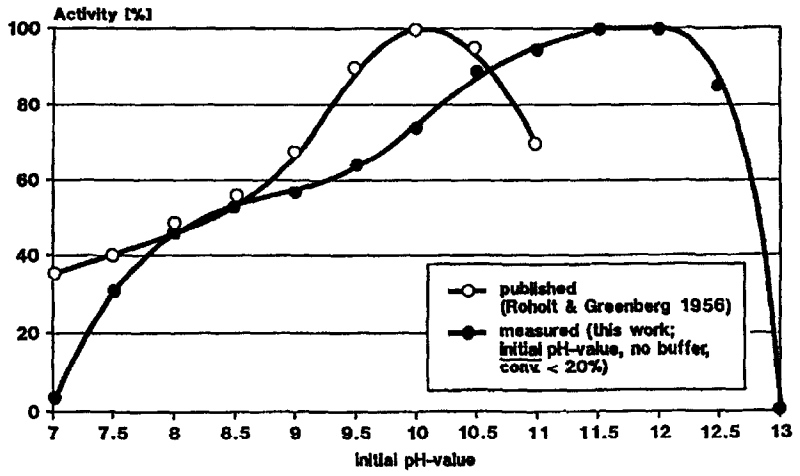


Figure 2. Activity vs pH profile of arginase.[4]

For all conversion vs time profiles, the concentration of L-Arg was varied without the addition of buffer. The enzyme/substrate (L-Arg)-ratio was kept constant at 10 kUnits/(L×M L-Arg). Thus, the profiles in Figure 3 should all superimpose; deviations might be caused by pH variation at different substrate concentration and inhibition effects.

It can be inferred from the curves in Figure 3 that L-Arg is not a substrate inhibitor. Since, especially at intermediate conversions, the rate decreases with increasing [L-Orn] but still reaches 100 % conversion at long times the product most likely acts as a competitive inhibitor, in accordance with the literature.[16] The reaction rate with L-Arg is also decreased in the presence of equal amounts of D-Arg (racemate). Attainment of complete conversion in each case also suggests competitive inhibition. By nonlinear regression of the initial rate data with L-Arg, K_m was determined as 25.5 mM, V_{max} as 459 Units [= μmol/(min mg)]. With typical [E] of 0.46 μM (MW = 120 kD), k_{cat} can be estimated as 1.2×10^3 s^{-1}. These data compare with K_m values of 4.0 mM[16] (29 mM for ox liver arginase)[26] and k_{cat} values of 6×10^3 s^{-1},[16] or 2.2×10^3 s^{-1},[17] (1.17×10^3 s^{-1} at pH 9.5[7] for the beef liver enzyme).

Inhibitors: L-Orn, L-Lys and D-Arg. The inhibitory effect of L-Orn and L-Lys impurities which are often found in raw Arg solutions was studied further by plotting initial rates of a 50 mM L-Arg solution with specified amounts of inhibitor at the usual enzyme/substrate-ratio (Table 2 and Figure 4).

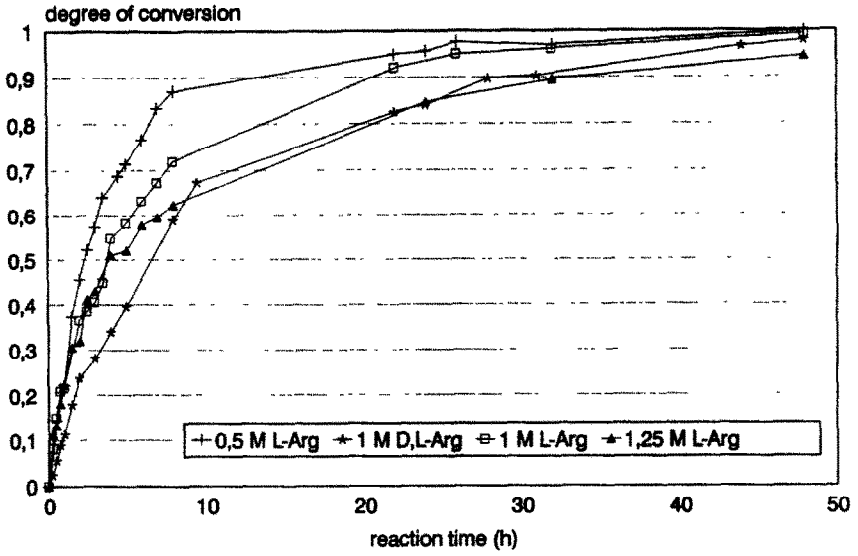


Figure 3. Conversion vs time profiles of the arginase reaction.

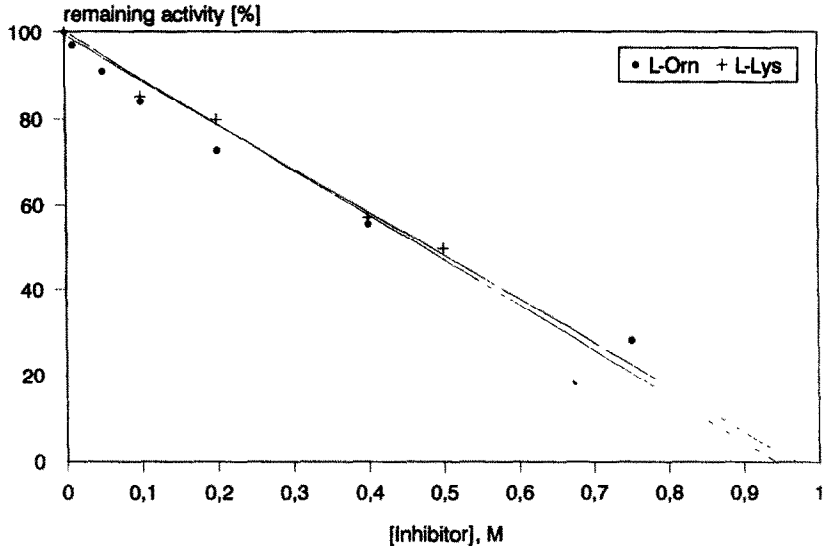


Figure 4. Inhibition of arginase by L-Orn and L-Lys.

Table 2. Effects of L-Orn and L-Lys on the hydrolysis of L-Arg

[L-Orn] (M)	conversion after 2 h (%)	$V_{o,Arginase}$ (mol/(L·h)	$V_{o,Arginase}$ (%)
0	40.2	0.151	100.0
0.01	39.0	0.146	97.0
0.05	36.6	0.137	91.0
0.1	28.5	0.107	70.9
0.2	24.1	0.090	60.0
0.4	22.3	0.084	55.5
0.75	11.3	0.042	28.1

[L-Lys] (M)	conversion after 2 h (%)	$V_{o,Arginase}$ (mol/(L·h)	$V_{o,Arginase}$ (%)
0	40.2	0.151	100.0
0.1	34.3	0.129	85.3
0.2	32.1	0.120	79.9
0.4	22.9	0.086	57.0
0.5	19.9	0.075	49.5
0.75	17.5	0.066	43.5

Cond. 5×10^{-4} M $MnSO_4 \cdot H_2O$, 2.5×10^{-4} M ascorbic acid, 50 mM L-Arg, $[E]$ = 100 kUnits/(L × M L-Arg), $[S]_o/[E] = 1.63 \times 10^6$, starting pH: 11.5.

A linear slope of a $1/[S]$ vs $[I]$ plot distinguishes a purely competitive inhibition mechanism from a partially competitive one.[33] Simultaneously, the apparent and the intrinsic K_m-value are proportional to $[I]$ according to eq. 4 from which the inhibition constant K_i can be calculated:

$$K_{m,app} = K_{m,intr}(1 + [I]/K_i). \quad (4)$$

Since the runs have been conducted at [L-Arg] = 50 mM = $2K_{m,intr}$, $V_o = V_{max}\ 2\ K_{m,intr}/\{K_{m,intr}(3 + [I]/K_i)\} = V_{max}\ 2/(3 + [I]/K_i)$. At $[I] = K_i$, $V_o = V_{max}\ 2/(3 + 1) = V_{max}/2$. So, K_i can be calculated with sufficient accuracy from a V_o vs $[I]$ plot such as Figure 4. For L-Orn, K_i is determined as 487 mM ($r^2 = 0.985$), for L-Lys, K_i equals 484 mM ($r^2 = 0.990$), i.e. L-Orn and L-Lys are equally strong inhibitors. These values are far higher than those in the literature: 1.3 mM.[6,16] At ordinary assay conditions, [L-Orn] << K_i, so inhibitory effects are not observed. In this work, however, maximum product concentration was sought for optimal space–time–yield and work-up efficiency.

D-Arg has been tried as a substrate but not checked as potential inhibitor. For the results in Table 3, [L-Arg] was kept constant at 500 mM while increasing amounts of D-Arg were added to cover the range from pure L-Arg to the racemate (D,L-Arg). While D-Arg certainly seems to inhibit the enzymatic hydrolysis of L-Arg, $K_{i,D\text{-}Arg}$ cannot be calculated according to the procedure applied for L-Orn and L-Lys because $[S]$ = 500 mM = 20 $K_{m,intr}$ and thus V_o is too close to V_{max}. A rough estimate from the initial rate data in Table 3 suggests that the inhibition constant for D-Arg might be around 600–800 mM, so even at the solubility limit of D,L-Arg (~ 1.25 M) inhibition by equimolar amounts of D-Arg is not important.

Selectivity of arginase

Enantioselectivity of arginase. D-Arg is neither a substrate of arginase nor a non-competitive inhibitor (Figure 3); the other main condition for using the arginase reaction for the preparation of D-Arg (eq. 2) is sufficiently high enantioselectivity of the enzyme. In a 50:50 mixture of D- and L-Arg, the formation of L-Orn as well as D-Orn was followed by chiral HPLC directly from the reacting solution at different degrees of conversion to test the enantioselectivity of calf liver arginase. At no degree of conversion could D-Orn be detected in the solution. To allow calculation of the E-value which characterizes enantioselectivity according to eq. 10^{27} the limit of detection of 1.0 % of D-Orn next to L-Orn was taken as the maximum possible D-Orn content. With $e.e._p$ = 0.98 at 50 % conversion (x = 0.5, based on D,L-Arg), E = 458, i.e. E > 100 is obtained from eq. 10. Since the *e.e.* of both isolated D-Arg and L-Orn products was higher than 99.8 %,

the above calculation of E represents a conservative estimate and calf liver arginase can be regarded as completely enantioselective.

Stability of arginase

Temperature stability. To assess the storage stability of arginase and to investigate the influence of temperature on the stability as well as the role of ascorbic acid as a stabilizer of arginase, deactivation curves were measured at different temperatures. The data in Table 4 reveal that, at all investigated temperatures in the range of –25 to 60 °C, calf liver arginase deactivates by a simple exponential decay law:

$$[E]_{\text{active},\,t} = [E]_{\text{active},0} \exp(-k_{\text{deact}}\, t). \quad (5)$$

The corresponding half-time of deactivation, $\tau_{1/2}$, after which 50 % of the initial activity remains, can be calculated by $\tau_{1/2} = \ln 2/k_{\text{deact}}$.

Below room temperature, arginase is stable over several months at pH 9.5 if both manganese ions and ascorbic acid are added. In the range of temperatures typical for processing, stability is sufficient ($\tau_{1/2}$ at 25 °C is 70 days). However, even at slightly elevated temperatures, arginase deactivates rapidly: $\tau_{1/2}$ at 37 °C is only nine days. Without the initial addition of ascorbic acid, the enzyme deactivates significantly faster. The stabilization of arginase at temperatures higher than 25 °C through the addition of ascorbic acid is reflected in a lower free enthalpy of deactivation $\Delta G^{\ddagger}$. The activation parameters $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are obtained from a $\ln (k_{\text{deact}}/T)$ vs $1/T$ plot (Figure 5) by taking logarithms of the expression for k_{deact} from the theory of absolute reaction rates

$$k_{\text{deact}} = (kT/h) \exp[-(\Delta G^{\ddagger})/RT]$$
$$(kT/h) \exp[-(\Delta H^{\ddagger} - T\Delta S^{\ddagger})/RT]; \quad (6a)$$

$$\ln (k_{\text{deact}}/T) = \ln (k/h) + \Delta S^{\ddagger}/R - \Delta H^{\ddagger}/RT. \quad (6b)$$

Table 3. Effect of D-Arg on L-Arg hydrolysis by arginase

Ratio L/D-Arg	Arg-conf.	conversion (%) after 1.0 h	1.5 h	2.0 h	$V_{o,\text{Arginase}}$ (mol/L/h)	$V_{o,\text{Arginase}}$ (%)
100:0	*L*	*37.4*	*40.7*	*45.6*	*0.187*	*100.0*
85:15	L(D)	25.1	34.8	40.2	0.150	80.3
70:30	L(D)	24.6	31.8	35.4	0.1380	73.8
60:40	L(D)	22.2	32.2	37.8	0.1376	73.6
50:50	*DL*	*15.6*	*19.4*	*26.4*	*0.097*	*59.5*

Cond. 5×10^{-4} M $MnSO_4 \cdot H_2O$, 2.5×10^{-4} M ascorbic acid, 0.5 M L-Arg, $[E]$ = 100 kUnits/(L × M L-Arg), $[S]_o/[E] = 1.63 \times 10^6$, starting pH: 11.5.

Table 4. Temperature stability of calf liver arginase

T (K)	T (°C)	with ascorbic acid k_{deact} (h^{-1})	with ascorbic acid $\tau_{1/2}$ (h)	without ascorbic acid k_{deact} (h^{-1})	without ascorbic acid $\tau_{1/2}$ (h)
253	-25	$4.5 \cdot 10^{-5}$	15359	-	-
277	4	$7.5 \cdot 10^{-5}$	9200	-	-
298	25	$4.1 \cdot 10^{-4}$	1682	-	-
303	30	$1.1 \cdot 10^{-3}$	645	$1.2 \cdot 10^{-3}$	573
310	37	$3.2 \cdot 10^{-3}$	217	$5.5 \cdot 10^{-3}$	125
323	50	$1.9 \cdot 10^{-2}$	36.5	$4.4 \cdot 10^{-2}$	15.6
333	60	$7.1 \cdot 10^{-2}$	9.8	$1.97 \cdot 10^{-1}$	3.52

Cond. 5×10^{-4} M $MnSO_4 \cdot H_2O$, if applicable 2.5×10^{-4} M ascorbic acid, 0.75 L-Arg, $[E]$ = 10 kUnits/(L × M L-Arg), $[S]_o/[E] = 1.63 \times 10^6$, starting pH: 9.5

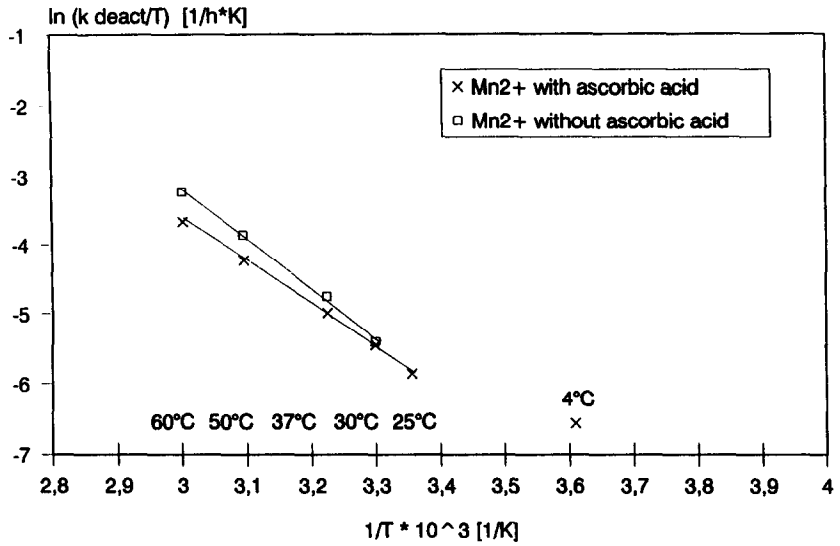


Figure 5. Temperature stability of calf liver arginase [$\log(k_{deact}/T)$ vs $1/T$ plot].

From the slopes of the ln (k_{deact}/T) vs $1/T$ plot, $\Delta H^{\ddagger}$ in the presence of ascorbic acid is –117.1 ± 2.4 kJ/mol (25–60 °C; r^2 = 0.9988), if stabilizer is absent $\Delta H^{\ddagger}$ equals –138.1 ± 5.2 kJ/mol (30–60 °C; r^2 = 0.9972); from the intercepts, $\Delta S^{\ddagger}$ is calculated to be +15.8 ± 0.7 J/mol K in presence and +87.7 ± 1.2 J/mol K in absence of ascorbic acid. At 298 K (25 °C), the difference in $\Delta G^{\ddagger}$ is minimal –112.0 (no ascorbate) vs –112.4 kJ/mol while at 333 K (60 °C), $\Delta\Delta G^{\ddagger}$ is substantially larger at 2.9 kJ/mol (–108.9 vs –111.8 kJ/mol). The data for k_{deact}/T at temperatures lower than 25 °C in presence of stabilizer do not follow eq. 6, so that identity of the mechanism of deactivation most likely holds for $T \geq 298$ K only. The significance of stabilization of arginase by ascorbate for the operational stability of the enzyme during reaction cannot be shown by comparing $\Delta G^{\ddagger}$s but must be elucidated with a test of operational stability.

Operational enzyme stability. On both laboratory scale (10 mL) as well as pilot scale (12 L) operational stability of arginase was determined with enzyme-membrane CSTRs. Since replenishing lost enzyme activity would have meant the addition of very small amounts of enzyme on lab scale, both enzyme activity consumption per unit mass (kg) of L-Orn product as well as the deactivation rate constant k_{deact} under operating conditions were determined from conversion–residence time-profiles (Table 5). If active enzyme concentration at time t, $[E]_t$, is assumed to be proportional to degree of conversion at that time, x_t, then the value of k_{deact} can be calculated with the exponential decay law eq. 7):

$$x_{t2}/x_{t1} = [E]_{t2}/[E]_{t1} = \exp\{-k_{deact}(t_1 - t_2)\} \quad (7)$$

or

$$k_{deact} = -\{\log(x_{t2}/x_{t1})\}/(t_1 - t_2). \quad (8)$$

Enzyme consumption per unit mass of product (EC/kg L-Orn) is determined from the slope of conversion vs residence time at maximum conversion, U/h, as well as residence time τ, initial substrate concentration $[S]_0$ and maximum conversion x_{max}:

$$\text{EC (U/kg L-Orn)} = (\text{U/h})(1000/MW_{L\text{-Arg}})/(\tau\,[S]_0\,x_{max}). \quad (9)$$

Values for both k_{deact} and EC/product mass are listed in Table 5.

Table 5. Operational stability of arginase

Scale	agitation	stabilizer [Asc.a.](M)	pH	oper. stab. (U/kg L-Orn)	k_{deact} (h^{-1})
10mL	200/min	$5 \cdot 10^{-4}$	10.5	15800	$3.7 \cdot 10^{-2}$
10mL	200/min	$2.5 \cdot 10^{-4}$	9.5	5460	$3.8 \cdot 10^{-3}$
12L	recycle (τ = 3h)	0	9.5	5890	n.det.
12L	no	0	9.5	5000	n.det.
12L	no	$2.5 \cdot 10^{-4}$	9.5	270	n.det.

Cond. 5×10^{-4} M $MnSO_4 \cdot H_2O$, ascorbic acid as indicated, 0.75 M L-Arg, $[E]$ = 100 kUnits/(L × M L-Arg), $[S]_0/[E] = 1.63 \times 10^6$, indicated pH-value is pH of product solution measured at maximum conversion.

The numbers for k_{deact} at 25 °C from operational stability measurements (3.8×10^{-3} h^{-1}; Table 5) and temperature stability measurements (4.1×10^{-4} h^{-1}; Table 4) suggest that during the arginase reaction the enzyme is deactivated primarily by mechanical effects from stirring or, more likely, by irregular or insufficient supply of manganese activator and not by temperature effects. From the results in the pilot unit it is concluded that, while stopping mechanical agitation results in a decrease of enzyme deactivation, the stabilizing effect of adding ascorbic acid achieves the main improvement in lowering the consumption of enzyme units.

Other potential causes of arginase instability. Tests of arginase activity in a sequence of batches in the presence of polysulfone and regenerated cellulose membrane materials as well as no membrane for control revealed no difference in enzyme stability. Likewise, addition of large amounts of solid MnO_2 to arginase solution to test susceptibility to solid precipitates did not cause change in activity during a sequence of four batches.

Process configuration and scale-up

Since arginase was found to be sensitive to mechanical agitation it was decided to check the influence of agitation by employing either the usual recycle reactor arrangement with continuous pumping on the pilot scale or an alternative batch-UF-reactor configuration: the reaction was carried out in a quiescent medium to avoid mechanical agitation while the medium was conveyed hydraulically to charge the reactor and to separate the enzyme over a UF membrane after completion of a batch for re-use in the next batch (Figure 6). Since residence time could not be determined in this set-up, k_{deact} could not be calculated. Instead, enzyme unit consumption data based on the amount of L-Orn produced were taken for comparison with conditions on the lab scale.

Arginase in a pumped solution is found to be unstable at an initial pH of 9.5 with Mn^{2+} but no ascorbic acid added: conversion after 24 h decreased from 53 % to zero after five runs. Somewhat surprisingly, enzyme stabilized by half-equimolar addition of ascorbic acid to Mn^{2+} in the lab-scale reactor at pH 9.5 deactivated almost as fast as the unstabilized solution in the pilot-scale reactor (5460 vs 5890 U/kg L-Orn). A quiescent medium not containing any ascorbate protected the enzyme somewhat against deactivation, so that 8 % conversion (down from 60 %) remained after eight batches (5000 U/kg L-Orn). In contrast, during another run in a quiescent medium at pH 9.5 with half-equimolar amounts of ascorbic acid added compared to Mn^{2+}, the enzyme was almost stable (88–63 % conversion) within 12 runs (270 U/kg L-Orn).

Discussion and Conclusion

Calf liver arginase is sufficiently stable at temperatures up to about 37 °C but seems to be deactivated by several other factors besides high temperature. Comparison between temperature stability and operational stability data suggest that mechanical stress through stirring markedly accelerates deactivation as do pH values above 10. However, neither the presence of a UF membrane nor of solid MnO_2 causes deactivation of arginase.

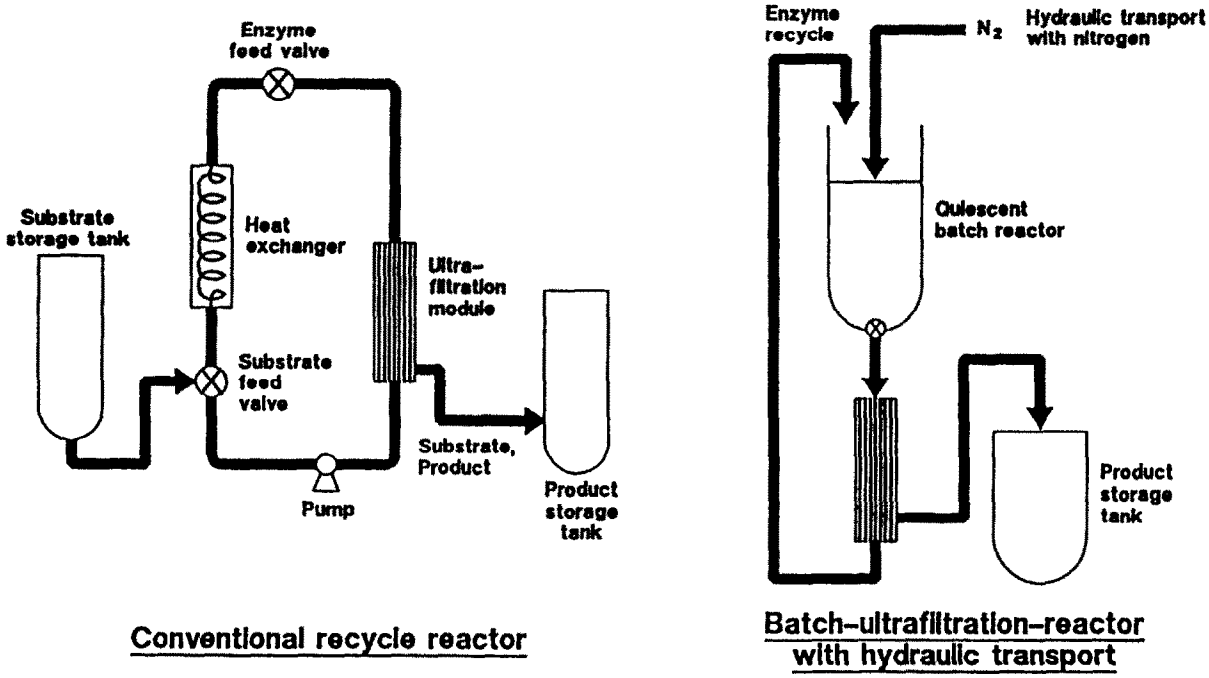


Figure 6. Process design of arginase reaction without mechanical agitation.

Temperature stability studies demonstrate the stabilizing effect of ascorbic acid on arginase. The reason for this effect, while not having been proven conclusively, most likely is the prevention of oxidation and concurrent removal from solution of the activator Mn^{2+} to solid MnO_2.

The results demonstrate the utility of calf liver arginase for hydrolysis of L-Arg for synthesis of L-Orn salts as well as of D,L-Arg for obtaining D-Arg besides L-Orn. While Mn^{2+}-ions are necessary and sufficient for activation, stabilization is required for sufficient enzyme operating life. Even on pilot scale, satisfactorily low values for enzyme consumption per kg L-Orn can be achieved. Scalability of the arginase reaction seems to present no particular problem.

Experimental Section

Materials

Calf liver arginase (activity 110 U/mg) was obtained from Boehringer Mannheim (Mannheim, Germany). Amino acids were from Rexim (Ham, France). $MnSO_4$, MnO_2 and ascorbic acid were from E. Merck (Darmstadt, Germany).

Batch conditions

Starting pH value, except for the activity–pH curve, was either 11.5, the natural value for L-Arg in water, or 10.0 (pH value of Orn in water), adjusted from pH 11.5 with conc. HCl. An initial pH of 11.5 was allowed to adjust freely between 11.5 and 10.0 during the arginase reaction (V_{max} does not change much in that range); no buffer was added to the solution because high salt concentrations are undesirable for downstream processing. The temperature was 25 °C unless otherwise noted. Typical concentrations of L-Arg were 0.75 M. Unless otherwise mentioned, $[Mn^{2+}] = 5 \times 10^{-4}$ M. Reaction progress was followed by HPLC [amino column, mobile phase: phosphate buffer (pH 6.4)/acetonitrile].

Kinetic data

Initial rate data were analyzed by a non-linear regression method (Marquardt routine) with the program OPTI (U. Giesecke, KFA Jülich).

Determination of enantioselectivity

The enantioselectivity of arginase was determined by measuring concentrations of D-Arg, L-Arg, D-Orn and L-Orn simultaneously by HPLC (Crownpak CR$^+$ column). Enantioselectivity of enzymatic reactions is expressed as E, given by the following equation:[27]

$$E = \frac{\ln\{(1-x)(1-e.e._S)\}}{\ln\{(1-x)(1+e.e._S)\}} = \frac{\ln\{1-x(1+e.e._P)\}}{\ln\{1-x(1-e.e._P)\}} = \frac{\ln\{[L\text{-Arg}]/[L\text{-Arg}]_0\}}{\ln\{[D\text{-Arg}]/[D\text{-Arg}]_0\}}, \quad (10)$$

where x = degree of conversion, and $e.e._S$ and $e.e._P$ = enantiomeric excess of substrate (Arg) and product (Orn), respectively. The E-value for a completely enantiounspecific reaction equals one; the higher the E-value the more enantiospecific the enzyme with regard to a pair of enantiomers of substrates and products.

Separation and work-up of D-Arg and L-Orn

After arginase reaction, the D-Arg/L-Orn solution (100 mL) was given on an ionic exchange column with basic resin (Lewatit M600) (resin volume 130 mL, height of bed 17 cm, capacity of column 0.7 mol OH$^-$/L resin). The Arg front elutes after about 50 mL at pH 11.5. The next 800 mL contained only Arg and urea according to HPLC analysis. The solution was clarified with charcoal and concentrated on a rotary evaporator to 20 mL. Arg was precipitated with 3 to 5 volumes of isopropanol, filtered, washed and dried under vacuum at 60 °C. The dried mother liquor also contains Arg as side product next to urea as main component.

Orn was eluted with 200 mL 5 % acetic acid at a pH of 9.9 to 8.5 (elution of Orn starts at about 150 mL). To form the acetate salt, equimolar amounts of glacial acetic acid were added (pH value reaches 6.9), the solution clarified with charcoal, and concentrated to 30 mL. L-Orn acetate was precipitated with 3 volumes of isopropanol and dried under vacuum at 60 °C.

Temperature stability

Arginase (30 U/mL) was added to 0.75 M L-Arg; the enzyme converts all substrate to product so that storage conditions closely mimic conditions during reaction. After complete conversion at 25 °C after 24 h, the enzyme solution was stored in water baths (± 0.1 °C) at the designated temperature and pH 9.5 for defined lengths of time. An aliquot was added to fresh 0.75 M L-Arg solution at pH 9.5 and 25 °C. Conversion was measured after 2 h and compared with the value at 0 h storage time.

Recycle reactor[28]

On the lab scale, an enzyme membrane reactor (volume 10 mL) with flat membrane (polysulfone) and stirred compartments was employed; residence times τ were between 2.5 and 4 h. On the pilot scale, a recycle reactor (volume 12 L) with a hollow-fiber UF module 2.5 m^2 (Romicon), polysulfone was used; residence time τ was 3 h. Reactions conditions were as mentioned above.

Operational enzyme stability

The basis for measuring operational enzyme stability has been described in refs[29,30]. Operational stability is conveniently measured in a recycle reactor operated as a CSTR with defined residence time τ, initial substrate concentration $[S]_0$, and initial enzyme concentration $[E]_0$.

In the course of the reaction, the enzyme deactivates, i.e. $[E]$ decreases. To keep the degree of conversion constant,

either fresh enzyme has to be added, effectively bringing [E] back up to $[E]_0$, or τ has to be increased to compensate for the decrease in active enzyme concentration due to inactivation. Choosing the first strategy, enzyme stability can be expressed as the amount of enzyme (in units or weight) consumed per unit mass of product, EC/unit mass of product.

Acknowledgments

The authors would like to thank Mrs G. Geiger, Mess H. Both, H. Werner and D. Orzewski for laboratory support as well as Dr K. Günther and Mr S. Merget for analytical support with measurements of enantioselectivity.

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(*Received* 7 *February* 1994; *accepted* 31 *March* 1994)