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AGMATINE COUMAROYLTRANSFERASE FROM BARLEY SEEDLINGS

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Key Word Index—*Hordeum vulgare*; Gramineae; barley seedlings; biosynthesis; cinnamic acid amides; coumaroylagmatine; hordatines; coenzyme A.

Abstract—4-Coumaroyl-CoA: agmatine *N*-4-coumaroyltransferase (agmatine coumaroyltransferase; ACT) from barley seedlings was purified with a 100-fold increase in specific activity and a 65% yield. Its MW was 40 000 and it was shown to be specific for agmatine as the amine substrate. The coenzyme A derivatives of cinnamic, coumaric, caffeic, ferulic and sinapic acids would all act as substrates, in diminishing order of activity.

INTRODUCTION

Dimers of coumaroylagmatine known as the hordatines which are present in barley seedlings [1–3] are thought to have a role in the protection of the plant against fungal infection [4]. An enzyme which synthesizes coumaroylagmatine from agmatine and coumaroyl-CoA [5] has been detected in extracts of 3-day-old barley seedlings (Scheme 1). The present communication reports the partial purification and some properties of this enzyme, which is known as 4-coumaroyl-CoA: agmatine-*N*-4-coumaroyltransferase (agmatine coumaroyltransferase, ACT) (EC 2.3.1.—).

RESULTS AND DISCUSSION

In earlier work [5], ACT was detected by a radioisotope assay using [U-^{14}C]agmatine, and separation of the products by TLC. A more rapid spectrophotometric assay has now been developed which is similar to that used for hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase [6]. Coumaroyl-CoA has a λ_{max} at 333 nm and coumaroylagmatine has λ_{max} at 292 and a shoulder at 304 nm. The depletion of coumaroyl-CoA during the ACT reaction was measured by the decrease of the absorbance at 333 nm. A linear relationship was found between ΔA_{333} and enzyme concentration. Approximate extinction coefficients ($\Delta\varepsilon$) were calculated for the synthesis of agmatine conjugates from coumaroyl-CoA and some other cinnamoyl-CoA derivatives (Table 1).

The enzyme was completely soluble; after centrifugation at 100 000 *g* for 1 hr, all of the ACT activity was in the supernatant. A five-step procedure for the purification of ACT from frozen shoots of barley seedlings is shown in Table 2. This resulted in a 100-fold increase in specific activity with a 65% yield. The majority of the ACT was retained by the immobilized agmatine affinity column (AC-Ultrogel ACA 34) when it was applied in dilute solution at a slow flow rate (4 ml/hr per cm^2). ACT was desorbed from the column in a sharp band by agmatine at concentrations between 1 and 2 mM. The ACT binding

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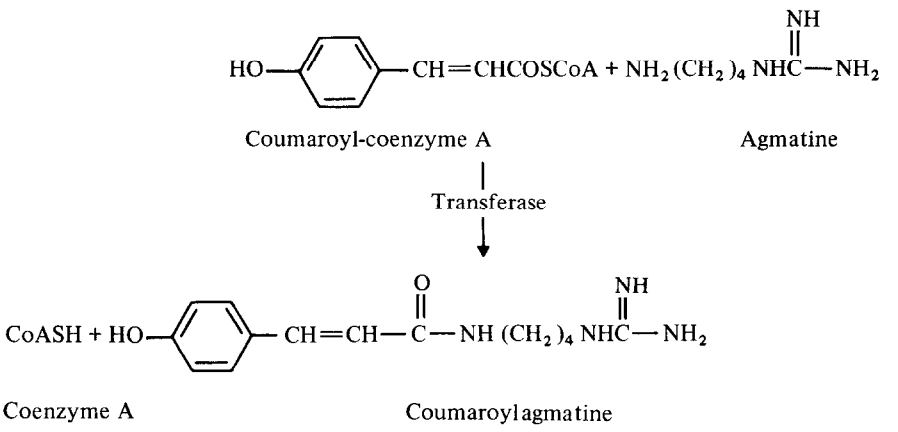

Scheme 1. Reaction catalysed by ACT.

Table 1. Approximate extinction coefficient values ($\Delta\varepsilon$) and kinetic data for the conversion of cinnamoyl-CoA derivatives to the appropriate agmatine amide by ACT

Substrate	λ_{max} (nm)	$\Delta\varepsilon$ ($M^{-1}\,cm^{-1}$)	Apparent K_m (μM)	V_{max} (nkat/g fr. wt)
Cinnamoyl-CoA	311	1.43×10^4	3.3	10.6
Coumaroyl-CoA	333	1.21×10^4	1.5	1.60
Caffeoyl-CoA	346	0.63×10^4	3.3	0.83
Feruloyl-CoA	346	1.26×10^4	1.3	0.70
Sinapoyl-CoA	349	1.3×10^4	50.0	0.64

Table 2. Purification of ACT from 10 g frozen barley seedlings

	Total volume (ml)	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg protein)	Yield (%)	Purification (-fold)
1. Centrifuged crude extract	31.5	16.6	81.9	0.20	100	1
2. Protamine sulphate	31.5	17.3	44.9	0.38	104	1.89
3. $(NH_4)_2SO_4$ (0–60%)	5.1	15.8	37.4	0.42	95	2.08
4. Gel filtration	83.5	18.6	12.0	1.55	112	7.6
5. Affinity chromatography (AC-Ultrogel ACA 34)	26.5	10.9	0.53	20.4	65.5	100

capacity of the affinity column declined substantially each time it was used. However, the affinity matrix still gave a positive reaction with the Sakaguchi test for guanidino groups, indicating that agmatine remained attached to the matrix. Nucleic acids are known to interact with polyamines immobilized on agarose [7, 8], and precipitation of nucleic acids from the ACT extract with protamine sulphate prior to affinity chromatography increased the ACT binding capacity of the column in subsequent purifications. Purification of ACT on DEAE-Sephacel with elution by a 0–0.5 M KCl gradient was inferior to that obtained by affinity chromatography.

The Bio-Gel A-0.5 m agarose column was calibrated for MW determination with five standard proteins. The estimated MW of both purified and unpurified ACT was 40 000. Activity of ACT was maximal at pH 7.5 and half-maximal activity occurred at pH 8 and 7. ACT was about twice as active in Tris buffer as in Bicine buffer. A mercaptan was found to be essential for preserving ACT activity during purification, and the optimal concentration of 2-mercaptoethanol was 25 mM. However, thiols will react spontaneously with coumaroyl-CoA with a resulting decrease in A_{333} (Negrel, J., unpublished results). In order to minimize this effect, the concentration of 2-mercaptoethanol in the spectrophotometric assay was kept below 3 mM, the actual concentration depending on the volume of enzyme extract added to the incubation mixture.

The apparent K_m of ACT for agmatine at pH 7.5 and 10 μM coumaroyl-CoA was 2.4 μM in crude extracts and 9.5 μM in purified extracts. This suggested a relatively high affinity of ACT for agmatine, but the affinity was reduced by purification, indicating some modification of the enzyme. ACT showed absolute specificity for agmatine as the cinnamic acid acceptor. No activity (< 0.1% of activity with agmatine) could be detected when the following were used as potential substrates: putrescine, *N*-carbamoylputrescine, spermidine, spermine, cadaverine, arginine, homoarginine and homoagmatine.

ACT was not specific for coumaroyl-CoA. Table 1 shows the apparent K_m and V_{max} values for some cinnamoyl-CoA derivatives at pH 7.5 and 0.2 mM agmatine. The apparent K_m values for the CoA derivatives of cinnamic, coumaric, caffeic and ferulic acids show similar high affinities of ACT for each substrate; however, the apparent K_m for sinapoyl-CoA was much greater, indicating that ACT has less affinity for this ester. The V_{max} values decreased with increased hydroxylation and methoxylation of the aromatic ring. Other enzymes which use cinnamoyl-CoA derivatives as substrates do not have such a broad specificity. In potato tubers, isoenzymes of hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase have been found, each with different specificity for cinnamoyl-CoA derivatives [9]. However, the occurrence of isoenzymes of ACT has not been disproved.

The close structural similarity of the hordatines to the synthetic fungicide guazatine and to the antibiotic eulicin suggests that the terminal guanidino groups are essential for anti-fungal activity [10]. Compounds analogous to the hordatines but containing putrescine in place of agmatine would not contain guanidino groups and would probably show much less anti-fungal activity; this probably accounts for the high specificity of the enzyme for agmatine. The relatively broad specificity for the acid moiety may reflects a less important role for this part of the hordatine molecule in fungitoxicity.

Since hordatine B appears to be formed structurally by condensation of one molecule of coumaroylagmatine with one molecule of feruloylagmatine [4] it was thought possible that free feruloylagmatine might also occur in the barley seedlings. This amide was synthesized by alkaline fusion [1], purified and used as a marker in paper electrophoresis. However, although coumaroylagmatine

could be easily detected, no evidence could be found for the presence of feruloylagmatine in the plant extract.

Amides of di- and polyamines with cinnamic acid and related hydroxyphenyl propenoic acids occur frequently in higher plants [10, 11] and a similar enzyme may be responsible for their formation.

EXPERIMENTAL

Barley (*Hordeum vulgare* L., cv Proctor) was grown in the dark [5]; the shoots were harvested 3 days after sowing, frozen in liquid N_2 and stored at $-40°$. Coumaroyl-CoA was synthesized chemically [5, 12]. [Guanidino-^{14}C]agmatine was synthesized enzymatically [5]. The affinity media were prepared according to the manufacturer's instructions.

Spectrophotometric assay. A mixture containing 100 μmol Tris, pH 7.5, 1 μmol EDTA, < 3 μmol 2-mercaptoethanol and enzyme in a final vol. of 1 ml was incubated at 30°; 10 nmol (10 μl) 4-coumaroyl-CoA was added and the decrease of A_{333} due to reaction with mercaptoethanol was recorded for 2 min. The ACT reaction was initiated by adding 200 nmol (20 μl) agmatine sulphate and the decrease of A_{333} was recorded for 4 5 min, during which time the reaction rate was almost linear, and the initial rate was calculated by subtracting the rate of loss of A_{333} due to 2-mercaptoethanol. The extinction coefficients for cinnamoyl-CoA derivatives in [12] were used to calculate the approximate extinction coefficients for the formation of cinnamic acid agmatine amides. The identity of the product was confirmed by using [guanidino-^{14}C]agmatine, and TLC of the spectrophotometric assay products in two solvent systems [5]. A single radioactive spot which co-chromatographed with coumaroylagmatine was detected.

Purification of ACT. All work was done between 0 and 4°. Two buffers were used: A, 0.1 M Tris, pH 8.5; B, 0.1 M Tris, pH 7.5; both contained EDTA (1 mM) and 2-mercaptoethanol (25 mM). Frozen barley shoots (20 g) were homogenized in 60 ml buffer A, squeezed through 4 layers of muslin and centrifuged at 27 000 *g* for 30 min. Protamine sulphate (15 mg as a 5% soln) was added dropwise to the stirred supernatant. After 30 min the mixture was centrifuged at 27 000 *g* for 30 min. Protein was precipitated from the supernatant by $(NH_4)_2SO_4$ at 60% saturation and redissolved in 4 ml buffer B containing 200 mg sucrose. The enzyme was applied to a Bio-Gel A-0.5 m column (3.5 × 55 cm) which had been pre-equilibrated with buffer B. The column was developed at 4 ml/hr per cm^2 and 5 ml fractions were collected. The ACT fractions (V_e 330 ml) were combined and pumped (4 ml/hr per cm^2) onto either a CH-Sepharose 4B-agmatine (Pharmacia) column (2.5 × 6.4 cm) or a AC-Ultrogel ACA 34-agmatine (LKB) column (2.5 × 4.2 cm), both of which had been pre-equilibrated with buffer B. The unadsorbed protein was eluted with 4 column vols. of buffer B; the column was then inverted and the buffer flow reversed before eluting ACT with a linear gradient of 0–10 mM agmatine. The combined ACT-containing fractions were concd by ultrafiltration with an Amicon UM10 membrane. The agmatine was removed from the conc. enzyme by dialysis against 3 × 200 vols. buffer B and the ACT was stored at −15°. The purified enzyme was used for all the kinetic studies.

Protein was determined by the method of ref. [13] with bovine serum albumin as standard. The MW of ACT was determined on the Bio-Gel A-0.5 m column, which was calibrated with standard proteins: cytochrome *c*, myoglobin, ovalbumin, bovine serum albumin (monomer and dimer) [14].

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