Aliphatic l-Amino Acid Decarboxylase from Ferns (Filicopsida)

Thomas Hartmann, Klaus Bax, and Renate Scholz

Institut für Pharmazeutische Biologie der Technischen Universität Braunschweig, Mendelssohnstr. 1, D-3300 Braunschweig, Bundesrepublik Deutschland

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A screening of 27 fern species (Filicopsida) out of 9 families revealed that 25 species were able to decarboxylate l-leucine to 3-methylbutylamine (isoamylamine). The enzyme of *Polypodium vulgare* has partially been purified and characterisized. All attempts to solubilize it from acetone preparations failed; however, approx. 50% of total activity could be extracted from dry material in the presence of detergents at high concentration. The soluble enzyme was purified 132-fold. l-Methionine was found the best substrate followed by norvaline, leucine, norleucine, isoleucine, homocysteine, valine. It has been confirmed that these substrates are decarboxylated by a single enzyme. The pH-optimum was at pH 5.0 (particulate preparation) and pH 4.5 (soluble enzyme). Decarboxylation is dependent on pyridoxal-5′-phosphate (PLP). A strictly substrate dependent coenzyme dissociation was observed which could largely be prevented by addition of 2-oxo-acids, such as glyoxylate or pyruvate. Apodecarboxylase prepared by prolonged substrate incubation was found to be extremely labile at pH 4.5 but stable at pH 6.5. A comparison of the fern enzyme with bacterial valine decarboxylase (EC 4.1.1.14) and leucine decarboxylase of red algae revealed great similarities especially in substrate specificity. It is suggested to unify these activities as "aliphatic l-amino acid decarboxylase".

Introduction

Lower aliphatic monoamines occur widely distributed in the plant kingdom [1]. Two alternative enzymatic routes of amine synthesis have been established: aldehyde amination and amino acid decarboxylation. The first route catalyzed by an alanine: aldehyde aminotransferase is the common biosynthetic pathway for a wide range of amines in higher plants [2–4], where this enzyme seems to be universally distributed irrespective whether a plant produces amines or not [5]. The transaminase has also been demonstrated in bacteria [6, 7]. Enzymes producing aliphatic monoamines by amino acid decarboxylation are the bacterial valine decarboxylase (EC 4.1.1.14) and the algal leucine decarboxylase. Both enzymes have in common a rather broad substrate specificity. Besides valine and leucine about 7 to 10 related amino acids are decarboxylated. The bacterial enzyme was characterized from *Proteus vulgaris* [8] and *Bacillus sphaericus* [9], the algal enzyme from various species of Rhodophyceae (red algae) [10–12].

In the present communication we report on the occurrence and partial purification of an amino acid decarboxylase from ferns which displays similar properties as the algal and bacterial enzymes mentioned above.

Reprint requests to Prof. Dr. T. Hartmann.
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Materials and Methods

Plant material

Most fern species were taken from their natural habitats or were grown in the Botanical Garden. Harvested samples were either directly freeze-dried or stored at −18° until use. *Polypodium vulgare* was collected in kg amounts in habitats near Altenahr (Rheinland) and Ameland (Holland). Fern gametophytes were grown on moist peat in a green house.

Acetone powder preparation

Fresh or deep-frozen fronds (midveins removed) of *Polypodium vulgare* were homogenized in 5 vol of acetone (−18°). Residue was collected by filtration under reduced pressure and extraction was repeated twice in the same way. The resulting powder was lyophilized, coarse particles were removed by passage through a sieve (pore size 0.4 mm) and the final product was kept dry and frozen at −18° until use; it can be stored without loss of activity for years.

Acetone powder could be further purified by extensive extraction with 0.2 M phosphate buffer, pH 7.0 at 4° without considerable loss of decarboxylase activity.

Enzyme extraction and partial purification

Lyophilized fronds of *Polypodium vulgare* were pulverized and passed through a sieve (pore size 0.4 mm). The dry powder can be stored at −18°; 5 g were extracted in 100 ml buffer consisting of 0.02 M phosphate buffer (pH 8.0), 0.35 M KCl, 0.1 mM diethyldithiocarbamate and 1 ml Triton-X-100. The suspension was stirred at 4° for 45 min. The residue was removed by centrifugation (30 000 × *g*/15 min), the supernatant was referred to as "crude extract".

This crude extract was made up 12.5% (w/v) with insoluble polyvinyl-pyrrolidone (Divergan-CE-5018, BASF, Ludwigshafen) and stirred for 30 min. The precipitate was removed by centrifugation and the supernatant was made up to 40% saturation with Butanol-1 at −18° added drop by drop and continuous stirring. The mixture was stirred for an additional 30 min, centrifuged (30 000 × *g*/15 min) and the aqueous layer was recovered. Residual butanol and low molecular compounds were removed by Sephadex-G-50 gel filtration. This step was combined with a buffer change to 50 mM Tris/HCl (pH 7.5) if a further purification by ion exchange chromatography was intended.

The desalted enzyme solution was bound to a column (1.2 × 7 cm) of DEAE cellulose (Servacel DEAE-23-SH; 0.93 meq/g) equilibrated with 50 mM Tris/HCl (pH 7.5). The decarboxylase was eluted with a linear gradient of KCl (0–0.6 M), total vol. 200 ml.

Preparation of apoenzyme

Purified enzyme (10 ml, approx. 100 nkat) was mixed with 12.5 ml 0.4 M phosphate/citrate buffer (pH 6.0) containing l-methionine (final assay concentration 60 mM). The mixture was incubated at 37 °C for 6 h, then placed in an ice-bath and pH was adjusted to 7.5. After centrifugation (19 000 × *g*/10 min) the supernatant was dialyzed against 0.01 M Tris/HCl buffer (pH 7.5) for 18 h. The dialysate was centrifuged and the supernatant kept cool (0–4°) until use.

Decarboxylase assay

Reactions were carried out in a Warburg apparatus at 37 °C according to ref. [10]. Standard assay (total vol. 2.0 ml): 200–300 µmol K-phosphate/Na-citrate at appropriate pH; 2 µmol pyridoxal-5′-phosphate (PLP); 80 µmol L-methionine (or alternative amino acid); 5 to 50 mg acetone powder or dry powder or 0.1–1.0 ml soluble enzyme in reaction buffer. The reaction was started by tipping in the substrate solution from the side-arme.

Amine estimation

In some studies amino acid decarboxylation was followed quantitatively by amine estimation according to the method given in ref. [21]. The amine was recovered from the reaction mixture by alkaline steam destillation. Following reaction with 2,4-dinitrofluorobenzene the resulting 2,4-dinitrophenyl derivatives were measured photometrically after TLC separation. The same chromatographic procedure was applied for quantitative amine separation and identification.

Proteine determination

The protein was estimated by the Lowry method [22].

Results

Distribution of the enzyme in ferns

Using the sensitive amine assay acetone powder preparations of the following 27 fern species out of 9 families (taxonomy according to ref. [13]) were screened for their ability to decarboxylate leucine to 3-methylbutylamine:

Aspidiaceae: *Dryopteris borreri*, *D. carthusiana*, *D. dilatata*, *D. filix-mas*, *D. villarii*, *Gymnocarpium dryopteris*, *G. robertianum*, *Polystichum aculeatum*, *P. braunii*, *P. lonchitis*, *P. setiferum*.

Aspleniaceae: *Asplenium ruta-muraria*, *A. trichomanes*, *A. viride*, *Phyllitis scolopendrium*.

Athyriaceae: *Athyrium distentifolium*, *A. filix-femina*, *Cystopteris fragilis*, *C. montana*, *Mattheuccia struthiopteris*.

Blechnaceae: *Blechnum spicant*.

Hypolepidaceae: *Pteridium aquilinum*.

Ophioglossaceae: *Botrychium lunaria*.

Osmundaceae: *Osmunda regalis.*
Polypodiaceae: *Polypodium vulgare.*
Thelypteridaceae: *Thelypteris limbosperma, T. palustris.*

With the exception of two (*Athyrium distentifolium; Cystopteris montana*) all species gave positive results. Preliminary experiments revealed that methionine and norvaline are most actively decarboxylated. With both substrates comparably high decarboxylase activities were determined monometrically for a variety of species (Table I).

The fern sporophylls contained decarboxylase activity at all developmental stages. *Phyllitis scolopendrium* sporophylls displayed the highest enzyme activity in just fully expanded fronds with beginning sporangia formation. The activity decreased slightly during leave maturing. No activity could be measured in rhizomes, roots but gametophytes and even spores of two species tested showed low but significant activity (Table II).

The characterization of the decarboxylase was performed with *Polypodium vulgare* since this plant is easily available and displayed high enzyme activity (Table I).

Purification

One of the unique properties of the fern decarboxylase is its solubilization behaviour which is similar to that of agal leucine decarboxylase [10]. But contrary to the agal enzyme which could not be extracted at all, we succeeded to solubilize the *Polypodium* enzyme at least partially from freeze-dried material in the presence of detergent at high concentration (Table III). Triton-X-100 (1%) gave the best results. Essentially no soluble activity could be detected when acetone powder was used instead of dry material (Table III). Under the experimental conditions applied approximately 50% of activity

Table I. Amino acid decarboxylation by crude enzyme preparations of sporophylls from various fern species.

Species	Decarboxylase activity (nkat/g a. p.)	
	Methionine	Norvaline
Asplenium ruta-muraria	71	52
Blechnum spicant	14	14
Dryopteris borreri	13	n. d.
dilatata	41	23
filix-mas	21	24
Phyllitis scolopendrium	25	19
Polypodium vulgare	254	79
Polystichum aculeatum	66	21
braunii	67	46
lonchitis	55	25
setiferum	39	16

a. p. = acetone powder. n. d. = not determined.
Enzymic activity was assayed manometrically at pH 5.0 using the standard assay conditions.

Table II. Decarboxylase activity in the different fern generations.

	Methionine decarboxylation [%]
Polypodium vulgare	
Sporophyte (sporophyll)	100
Gametophyte	2.2
Spores	1
Phyllitis scolopendrium	
Sporophyte (sporophyll)	100
Gametophyte	7.7
Gametophyte + young sporophytes	15.8
Spores	7.3

Enzymic activity was determined manometrically (standard assay). 100% activity: *P. vulgare* = 170 nkat/g acetone powder; *P. scolopendrium* = 26 nkat/g acetone powder.

Table III. Solubilization of decarboxylase activity from dry powders and aceton powders of *Polypodium vulgare.*

Extraction procedure	Methionine decarboxylation (%)[a]			
	Dry powder		Acetone powder	
	Soluble activity	Particulate activity	Soluble activity	Particulate activity
I. Extraction				
30 min	45	61	0	98
60 min	53	54	0	103
60 min (-Triton)	0	102	n. d.	
II. Extraction				
60 min	< 2	46	n. d.	

[a] Activity of the starting material (dry powder or acetone) powder was set 100.

I. Extraction: 2 g dry powder or acetone powder were extracted with 40 ml extraction buffer as given in Experimental. The supernatant represents the "soluble activity" the lyophilized residue the "particulate activity".
II. Extraction: the residue of the I. Extraction (60 min) was reextracted in the same way as given above.

could be extracted. The residual activity remained stable but firmly bound to the particulate material. The soluble enzyme activity was partially purified. The result of the purification procedure is summarized in Table IV. A butanol extraction was necessary to remove large quantities of lipophilic material which had been extracted in presence of the high levels of detergent. In combination with ion exchange chromatography this 3-step purification procedure gave enzyme preparations with a specific activity of approx. 180 nkat/mg protein which was found suitable pure for further enzyme characterization.

Some general properties

Under standard assay conditions using 0.2 M phosphate/citrate buffer the decarboxylase of *P. vulgaris* dry powders or acetone powders showed a pH-optimum at pH 5.0 (half-maximal activity at pH 4.5 ± 0.3 and 6.0 ± 0.2, respectively). In partially purified soluble enzyme preparations a shift of the optimal pH towards 4.5 was observed (half-maximal activity at pH 3.5 ± 0.3 and 6.0 ± 0.3, respectively).

Concomitant determination of amine (*n*-butylamine) formation and CO_2 production during norvaline decarboxylation revealed a 98% stoichiometry for CO_2 (mean value of 3 independent assays). Prolonged incubations of particulate and soluble enzyme preparations with [^{14}C-l]leucine revealed no evidence for interfering enzyme activities. No amino acid oxidase or amine oxidase activities could be measured manometrically.

Table IV. Purification of aliphatic amino acid decarboxylase from *Polypodium vulgare* sporophylls.

Purification step	Total activity [nkat]	Specific activity [nkat/mg protein]	Purification factor	Recovery [%]
Crude extract	626	1.37	1	100
Butanol extraction/ Sephadex-G 50	305	36.99	27	48
DEAE-cellulose	136	180.84	132	22

Substrate: l-methionine; preparation was started from 5 g sporophyll dry powder (total activity: 1.364 kat).

Coenzyme requirements

Methionine decarboxylation decreases rapidly with time unless PLP or Na-glyoxylate is added to the assays (Fig. 1 A, B). Once inactivated activity can be fully restored by subsequent addition of PLP, but only in assays performed at pH 6.0 (Fig. 1 B). Reactivation was incomplete if the same experiment was carried out at pH 4.5 (Fig. 1 A). No reactivation was observed when Na-glyoxylate was added instead of PLP. Preincubation of enzyme preparations in the absence of substrate did not influence enzyme activity (Fig. 1 C).

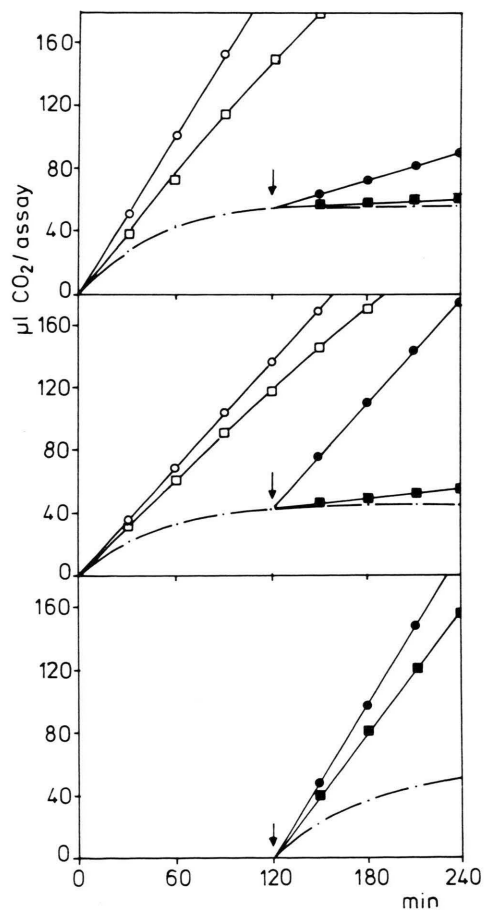

Fig. 1. Activation of methionine decarboxylation by PLP and Na-glyoxylate. A: Incubation under standard assay conditions at pH 4.5 with methionine (40 mM) as substrate and additives as indicated. B: as A, but at pH 6.0. C: as A, but methionine added at t_{120} min. PLP (1 mM) added at t_0 (○—○); PLP added at t_{120} min (●—●); Na-glyoxylate (1 mM) added at t_0 (□—□); Na-glyoxylate (1 mM) added at t_{120} min (■—■); without additives (·——·).

The results are comparable to those obtained with the algal leucine decarboxylase [10–12]: a) The dissociation of the holoenzyme appears to be strictly substrate dependent. b) Glyoxylate cannot substitute PLP as coenzyme but stabilizes the coenzyme-apoenzyme complex. To some extent in this function glyoxylate can be substituted by homologous 2-oxoacids such as pyruvate and 2-oxobutyrate. With higher homologous acids up to 2-oxooctanoate the effect, although still present, is increasingly masked by their action as competitive substrate inhibitors. 2-Oxoglutarate and oxaloacetate are ineffective.

The activatory function of 2-oxoacids is merely catalytical, as has been proved by experiments with [^{14}C]pyruvate. Table V summarizes the PLP and Na-glyoxylate concentrations needed for optimal decarboxylase activity. Added separately 1 mM PLP is necessary to obtain optimal activity. However, in the presence of 1 mM Na-glyoxylate the enzyme is nearly saturated at a 100-fold lower PLP concentration. This result indicate a K_m of PLP in the range of 10 µM [12].

The inability to reactivate the decarboxylase which previously had been resolved at pH 4.5, by addition of PLP (Fig. 1 A) indicates acid sensitivity of the apodecarboxylase.

To prove this idea apoenzyme was prepared with the procedure described in Experimental. The final preparation contained about 60% of original activity and represented almost pure apoenzyme (residual activity in the presence of 0.1 M glyoxylate $< 2\%$). As illustrated in Fig. 2 the apoenzyme is very acid sensitive. It remained entirely stable at pH 7.5 but more than 80% of activity were lost within 2 h at pH 4.5. The holoenzyme is stable under identical conditions. Any effort to stabilize the apoenzyme at acid conditions, *i.e.* addition of substrate, glyoxylate as well as dithioerithritol or other protective agents failed.

Substrate specificity

The activity of the decarboxylase with various substrate amino acids is summarized in Table VI. The decarboxylation pattern achieved with the 3 different enzyme preparations are quantitatively almost identical, suggesting the existence of one enzyme with a broad substrate specificity. This assumption is further supported by the following experimental observations:

Table V. Activation of methionine decarboxylation by various concentrations of PLP and Na-glyoxylate. Activity was determined under standard assay conditions (pH 4.5) with 40 mM methionine and additives as indicated.

Assay concentration [mM]		Methionine decarboxylation [µmol $CO_2 \cdot 2\,h^{-1} \cdot vessel^{-1}$]
PLP	Glyoxylate	
–	–	1.7
1.0	–	4.5
0.1	–	4.2
0.01	–	2.7
–	10.0	3.5
–	1.0	3.6
–	0.1	2.3
–	0.01	1.8
1.0	1.0	4.5
0.1	1.0	4.6
0.01	1.0	4.1

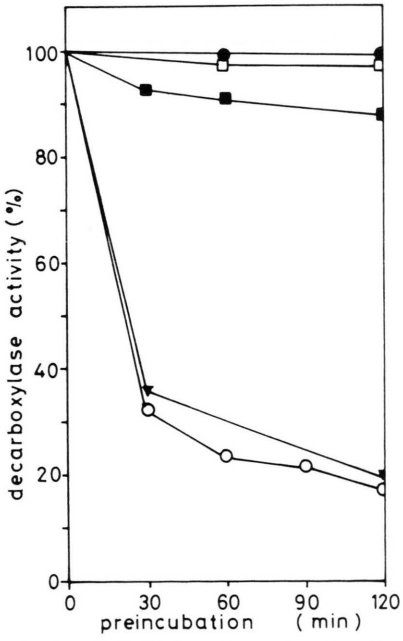

Fig. 2. pH-Stability of apodecarboxylase. Apodecarboxylase was preincubated in 0.2 M phosphate/citrate buffer as indicated. At intervals samples were removed and decarboxylase activity was determined in the presence of 1 mM PLP under standard assay conditions at pH 4.5. Substrate: 40 mM methionine. Preincubation at pH 4.5 (○—○); preincubation at pH 7.5 (●—●); preincubation in the presence of PLP (1 mM) at pH 4.5 (□—□); preincubation in the presence of Na-glyoxylate (1 mM) at pH 4.5 (▲—▲); preincubation at pH 4.5 and 0° (■—■).

a) When two substrates were added simultaneously no additive effects were found (Table VI).
b) Soluble enzyme preparations of different degrees of purity and activity displayed substrate patterns which are quantitatively identical.

Table VI. Substrate specificity of aliphatic amino acid decarboxylase in various preparations from *Polypodium vulgare*. Standard assay in the presence of PLP (1 mM) at pH 5.0 (acetone powder and dry powder) or pH 4.5 (purified enzyme).

Substrate (20 mM each)	Decarboxylase activity [%]		
	Aceton powder	Dry powder	Purified enzyme
Methionine	100	100	100
Norleucine	32	31	31
Leucine	31	32	34
Isoleucine	29	30	28
Norvaline	38	39	39
Valine	4	6	7
Aminobutyric acid[a]	< 2	< 2	< 2
Alanine[a]	< 2	< 2	< 2
Phenylalanine[a]	< 2	< 2	< 2
Homocysteine[b]	11	18	12
Methionine + isoleucine			57
Methionine + leucine			83
Methionine + norleucine			71
Methionine + norvaline			90
Leucine + norvaline			36
Norleucine + norvaline			36

[a] Amine assays positive.
[b] Figures not comparable; homocysteine reacts spontaneously with PLP yielding the respective thiazane derivative [12].

c) Apoenzyme prepared in the presence of methionine is not only inactive with methionine but also with the other substrate amino acids.
d) In the presence of norvaline or leucine partially resolved enzyme showed the same extent of inactivation for all substrates tested.
e) None-substrate amino acids, such as tyrosine and lysine did not facilitate coenzyme dissociation and thus were without any influence on decarboxylase activity with the various substrates.
f) The optimal pH of decarboxylation is the same with all substrates, *i.e.* pH 5.0 (dry and aceton powders) pH 4.5 (soluble enzyme), respectively.

D-Amino acids were not decarboxylated as well as 25 other L-amino acids including S-adenosylmethionine the substrate of well known bacterial and mamalian enzymes [14, 15].

Discussion

A particular property of the fern decarboxylase is its broad substrate specificity. Similar characteristics have been reported for bacterial valine decarboxylase [8, 9] and algal leucine decarboxylase [10–12]. The substrate patterns of the three enzymes, which are derived from distant and unrelated systematic groups of organisms, are virtually identical (Table VII). With the exception of phenylalanine which is a substrate of aromatic L-amino acid

Table VII. Comparison of the substrate patterns of "aliphatic amino acid decarboxylase" from *Proteus vulgaris* (valine decarboxylase) [8], *Bacillus sphaericus* (valine decarboxylase) [9], two red algal species (leucine decarboxylase) [10], and two fern species (this report). The relative substrate efficiency is arbitrarily expressed on the basis of leucine, which was set 100.

Substrate	Relative decarboxylase activity [%]					
	Bacteria		Rhodophyceae		Filicinae	
	Proteus vulgaris	*Bacillus sphaericus*	*Polysiphonia ureceolata*	*Cystoclonium purpurea*	*Polypodium vulgare*	*Phyllitis scolopendrium*
L-Leucine	100	100	100	100	100	100
L-Isoleucine	58	26	16	22	82	71
L-Norleucine	n. d.[a]	37	34	39	91	98
L-Valine	100	117	13	11	21	57
L-Norvaline	68	115	49	40	115	125
L-2-Aminobutyrate	32	62	12	13	< 5	38
L-Alanine	0[a]	9	3	< 2	< 5	< 5
L-Methionine	n. d.[a]	22	20	26	294	134
L-Phenylalanine	8	8	8	53	5	5

[a] Recent reaxamination revealed that the 3 amino acids are substrates [9].

decarboxylase (EC 4.1.1.28) [16] none of the amino acids listed in Table VII have been reported to function as substrates of specific decarboxylases. A specific methionine decarboxylase from *Streptomyces* sp. (EC 4.1.1.57) claimed by Hagino *et al.* [17, 18] has recently been purified [19]. This enzyme turned out to be an unspecific enzyme which in addition to methionine reacts with leucine, isoleucine, norleucine and norvaline, thus well fitting in the pattern outlined in Table VII.

From the quantitative point of view the substrate spectra listed in Table VII are different. Following the classical rules of enzyme nomenclature we must distinguish at least three enzymes: Bacterial valine (leucine) decarboxylase, algal leucine decarboxylase and a methionine decarboxylase from ferns. Within the red algae even more enzymes may be distinguished since several species were found which decarboxylate either methionine or phenylalanine with a higher efficiency than leucine [20]. To our opinion such classification would not satisfy the main characteristics of the decarboxylases, namely to display a broad specificity against a series of substrates. We suggest to comprise the activities as "aliphatic L-amino acid decarboxylase" analogous to "aromatic L-amino acid decarboxylase". This suggestion is further supported by other similarities existing between the individual enzymes. As many amino acid decarboxylases they are PLP-dependent. However, the strictly substrate dependent coenzyme dissociation seems to be a specific feature of aliphytic amino acid decarboxylase [8, 9, 10–12]. The algal and the fern enzymes display similar solubilization characteristics. The algal enzyme has not jet solubilized at all, the fern enzyme only incomplete from dry material. The activation of the fern and algal enzymes by glyoxylate and other 2-oxoacids is not known from other amino acid decarboxylases.

Acknowledgements

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