Inhibitors Influencing Plant Enzymes of the Polyamine Biosynthetic Pathway

Marbeth Christ, Hansruedi Felix, and Jost Harr

AGRO Research, Sandoz Ltd., CH-4002 Basel, Switzerland

Z. Naturforsch. **44c,** 49–54 (1989); received September 1, 1988

Triticum vulgare, S-Adenosylmethionine Decarboxylase, Arginine Decarboxylase, Ornithine Decarboxylase, Spermidine Synthase

Several enzymes involved in polyamine biosynthesis namely ornithine, arginine and S-adenosylmethionine decarboxylase as well as spermidine synthase, were analyzed in partially purified wheat extracts. For all enzymes effective inhibitors were found. Among them the most interesting was 1-aminooxy-3-aminopropane, which inhibited all three decarboxylases. Classical polyamine biosynthesis inhibitors like difluoromethylornithine, difluoromethylarginine, methyl glyoxal bis-(guanylhydrazone) and cyclohexylamine were also inhibitory on plant enzymes.

A remarkable difference in the amount of arginine and ornithine decarboxylase existed in wheat. Arginine decarboxylase seems to be more important at least during the early stage of development. Influence of polyamine synthesis inhibitors on polyamine levels is more likely to come from arginine decarboxylase inhibitors.

As inhibitors of all essential enzymes involved in plant polyamine biosynthesis were found, the study of the importance of polyamines in plant physiology will be considerably facilitated.

Introduction

Polyamines are widely distributed in both animals and plants. There is evidence to support the involvement of polyamines in growth and development [1, 2]. After several decades of intensive basic research, the metabolism of polyamines has apparently become a meaningful target for therapy in certain branches of clinical medicine [3]. In plants polyamine biosynthesis results from the activity of several enzymes: ornithine decarboxylase (the only putrescine-forming enzyme in animals), arginine decarboxylase, and in some species citrulline decarboxylase for putrescine synthesis; S-adenosylmethionine decarboxylase, spermidine synthase and spermine synthase for spermidine and spermine synthesis. In assays of ornithine and arginine decarboxylase decarboxylations not due to these enzymes were observed in plant extracts [4, 5]. S-Adenosyl-L-methionine seems to be labile in extracts [6–8]. Many compounds were synthesized for the inhibition of bacterial and animal enzymes [2], but only a few were screened for the inhibition of plant enzymes. In the present study enzyme assays were analyzed and used in testing numerous inhibitors mentioned in the literature. The aim was to find potent inhibitors of plant enzymes. It is not planned to obtain exact inhibitor constants, it is rather intended to use effective inhibitors in future experiments to elucidate the importance of polyamines in plants.

Abbreviations: ADC, arginine decarboxylase (EC 4.1.1.19); DFMA, α-difluoromethylarginine; DFMO, α-difluoromethylornithine; MGBG, methyl glyoxal bis(guanylhydrazone); ODC, ornithine decarboxylase (EC 4.1.1.17); SAMDC, S-adenosylmethionine decarboxylase (EC 4.1.1.50).

Reprint requests to Dr. H. Felix.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/89/0100–0049 $ 01.30/0

Experimental

Preparation of enzymes

Seeds of *Triticum vulgare* L. cv. Probus germinated in flats in vermiculite on a window sill with southern exposure at 22 °C. First leaves were sampled 5 days after germination. Thirty g of whole shoots were washed, dried with absorbent paper, frozen in liquid nitrogen and homogenized in a mixer at maximum speed for 30 seconds without adding a buffer. The homogenate was suspended in 12 ml 0.1 mM EDTA, 0.1 mM pyridoxal phosphate, 10 mM dithiothreitol and 200 mM sodium phosphate buffer pH 8.0 (buffer A) and centrifuged at 20,000 × *g* at 4 °C for 20 min. To remove all small particles the supernatant was passed over a nylon filter (52 μm). Protein was collected by an ammonium sulfate precipitation (70% saturation) and a centrifugation (20,000 × *g* at

4 °C for 10 min). The pellet was resuspended in 3 ml 0.05 mM EDTA, 0.05 mM pyridoxal phosphate, 2 mM dithiothreitol and 100 mM sodium phosphate buffer pH 8.0 (buffer B). The solution was desalted over a Sephadex G 25M column (PD-10, Pharmacia, Uppsala, Sweden). The column was preconditioned with 25 ml of buffer B, 2.5 ml of the enzyme solution were applied, the first fraction of 3.2 ml of the eluate was discarded and the next fraction of 3.5 ml was used for enzyme assays (5–7 g fresh weight per ml, 10–15 mg protein per ml). Portions of 0.6 ml were frozen at −20 °C; these aliquots were stable for at least 70 days without loss of activity.

Assays for enzymes

Enzyme activities were assayed in 100 µl reaction mixtures. The mixture for ADC contained 1 mM L-[U-^{14}C]arginine (5 mCi/mmol), 0.05 mM pyridoxal phosphate and 60 µl of the extract; the mixture for ODC contained 1 mM D,L-[1-^{14}C]ornithine (5 mCi/mmol) or 1 mM D,L-[5-^{14}C]ornithine (50 mCi/mmol), 0.05 mM pyridoxal phosphate and 50 µl of the extract; the mixture for SAMDC contained 0.5 mM L-[carboxyl-^{14}C]S-adenosyl-L-methionine (5 mCi/mmol) and 70 µl of the extract; the mixture for spermidine synthase contained 0.5 mM S-adenosyl-L-methionine and 0.1 mM [1,4-^{14}C]putrescine (50 mCi/mmol) and 70 µl of the extract. Incubations were carried out at 37 °C for 40 min. $^{14}CO_2$ release was followed in small plastic vials with stoppers pierced by a pin. Two pieces of filter paper (15 mm diameter) impregnated with 50 µl 2 M potassium hydroxide were attached to the pin inside the vial. The reaction was stopped by injection of 200 µl 12% (w/v) trichloroacetic acid. The vials were opened after 45 min of shaking (120 rpm) at 37 °C and the filter papers placed in scintillation vials. Radioactivity was determined in 10 ml scintillation fluid (Hydrocount™, J.T. Baker Chemicals B.V., Deventer, Netherlands) using a Tricarb 2000 CA scintillation counter (Packard, Canberra, Australia).

When reaction mixtures were subjected to thin layer chromatography (ODC with [5-^{14}C]ornithine as substrate and spermidine synthase), the reaction was stopped by adding 25 µl of a solution containing 0.7 mM putrescine, 0.7 mM ornithine and 35% (w/v) perchloric acid for ODC and 10 mM spermidine and 35% (w/v) perchloric acid for spermidine synthase. Amines in this mixture were derivatized with 250 µl dansylchloride (5 mg/ml acetone, freshly prepared) and 125 µl saturated sodium carbonate. The mixture was vortexed for 10 s and incubated in the dark for 16 h at room temperature. Unreacted dansylchloride was removed by adding 50 µl 0.87 M proline and incubation for 30 min. Dansylated amines were extracted with 0.25 ml benzene, 20 µl were applied to silica thin layer plates (Whatman LK 6DF, Whatman Chemical Separation Inc., Clifton, N.J., U.S.A.) and developed with chloroform and triethylamine (5:1, v/v) as solvents. Dansylated amines were visualized under UV light (254 nm), scraped off the plates and transferred into scintillation vials. The radioactivity was determined in scintillation fluid as previously described. The R_F value for ornithine was 0.29, for putrescine 0.54 and for spermidine 0.81.

Chemicals

Most chemicals were commercially available: S-adenosyl-L-methionine from Boehringer (Mannheim, F.R.G.), evernic acid and atabrine from SERVA (Heidelberg, F.R.G.), aminoguanidine and EDTA from Merck (Darmstadt, F.R.G.), MGBG from Aldrich (Steinheim, F.R.G.), Berenil (4,4′-diaminodiazoaminobenzene) from Calbiochem (La Jolla, CA, U.S.A.), hydrazinoornithine, 1,4-diaminobutan-2-one, α-methylornithine, phosphorylethanolamine, phosphorylcholine, canaline and ethambutol from Sigma (St. Louis, MO, U.S.A.), radiolabelled compounds from Amersham (England). All other compounds were from Fluka (Buchs, Switzerland) except for the following compounds that were synthesized in-house according to the references mentioned in Table II to V: NSD 1055 (4-bromo-3-hydroxylbenzyloxyamindihydrogen phosphate), 1-aminooxy-3-aminopropane, DFMO, trans-1,4-diamino-cyclohexane-1-carboxylic acid, DFMA, norspermidine, aminopropylcadaverine, aminopropylhexanediamine, bis(aminopropyl)-cadaverine, canavalmine and all hexahydropyrimidine derivatives.

Results and Discussion

In a first step enzyme assays were optimized and characterized. Approximate K_m values in semi-purified extracts were found to be as follows: 0.24 mM L-ornithine for the ODC, 0.48 mM L-arginine for the ADC and 17 µM S-adenosyl-L-methio-

Table I. Ornithine decarboxylase (ODC), arginine decarboxylase (ADC), S-adenosylmethionine decarboxylase (SAMDC) and spermidine synthase activities in *T. vulgare* extracts.

Enzyme	Substrate	Activity [nmol/h × *g* fresh weight] based on	
		$^{14}CO_2$ release	Thin layer chromatography
ODS	1 mM [1-^{14}C]ornithine	0.21	
	1 mM [5-^{14}C]ornithine		0.16
ADC	1 mM [U-^{14}C]arginine	5.3	
SAMDC	0.5 mM [carboxyl-^{14}C] S-adenosyl-methionine	0.93	
Spermidine synthase	0.5 mM S-adenosyl-methionine + 0.1 mM [1,4-^{14}C]putrescine	–	0.058

nine for SAMDC. During 40 min a linear increase in product formation was found for all enzymes. Amounts of extract *vs* product formation was linear up to 70 µl extract per assay. Because of potential problems with unspecific decarboxylation reactions [4, 5] enzyme activities were characterized as much as possible (Table I). ODC was measured by two methods: the standard $^{14}CO_2$-based assay and the demonstration of the labelled product putrescine. The total $^{14}CO_2$ produced was 30% higher than that due to ODC activities calculated from labelled putrescine found by thin layer chromatography in the incubation mixture. The $^{14}CO_2$ release not related to ODC activity was considerably lower than that found in other plant species [4, 5]. In both methods the ODC was inhibited by the specific suicide inhibitor DFMO. Most probably the difference in yield between the two assay methods did not originate from oxidative degradation of labelled putrescine, because amino-guanidine (0.2 mM), an inhibitor of this degradation, did not increase the production of putrescine from 5-[^{14}C]-D,L-ornithine (results not shown). ADC and SAMDC could be inhibited by specific inhibitors well known from the literature: DFMA reduced ADC activity drastically (Table II); MGBG inhibited SAMDC (Table III). Labelled spermidine, the product of spermidine synthase could be detected on thin-layer plates (Table I); the substrate for this enzyme, S-adenosyl-homocysteamine, was generated in the same reaction mixture by SAMDC.

Many inhibitors described in the literature were tested in our plant extract system. From the ODC inhibitors tested (Table IV) 1-aminooxy-3-aminopropane was the most active. A concentration of 0.06 µM still inhibited ODC activity by 50%. This compound was also rather inhibitory to ADC and SAMDC, thus allowing the control of all enzymes leading to putrescine and spermidine. Other good

Table II. Inhibition of ADC in extracts of *T. vulgare*. DFMO and DFMA were preincubated with the extract for 30 min without the substrate arginine. DFMO, an ODC inhibitor, was added to test the enzyme specificity of this compound. For each compound tested a reference for synthesis and further assays is given. An approximate I_{50}-value is given for active inhibitors.

Inhibitor	Concentration [mM]	Activity compared to control	I_{50} [µM]
Control	–	100	
DFMA [14]	0.1	9	9
	0.01	47	
	0.001	91	
Evernic acid [15]	1	74	
	0.1	97	
L-Canavanine-sulfate [16]	1	74	
	0.1	100	
NSD 1055 [17]	1	55	
	0.1	93	
1-Aminooxy-3-aminopropane [18]	1	8	400
	0.2	85	
	0.1	96	
DFMO [19]	0.5	100	

Table III. Inhibition of SAMDC in extracts of *T. vulgare*. For each compound tested a reference for synthesis and further tests is given. An approximate I_{50}-value is given for active inhibitors.

Inhibitor	Concentration [mM]	Activity in % as compared to control	I_{50} [μM]
Control	–	100	
MGBG [20]	1	5	14
	0.1	13	
	0.01	58	
	0.001	96	
1-Aminooxy-3-aminopropane [18, 21]	1	17	350
	0.1	86	
	0.01	100	
Berenil [22]	1	60	
Atabrine [23]	1	65	

Table IV. Inhibition of ODC in *T. vulgare* extracts by various compounds. DFMO and DFMA were preincubated with the extract for 30 min without the substrate ornithine. DFMA, an ADC inhibitor, was added to test the enzyme specificity of this compound. For each compound tested a reference for synthesis and further tests is given. An approximate I_{50}-value is given for active inhibitors.

Inhibitor	Concentration [mM]	Activity in % as compared to control	I_{50} [μM]
Control	–	100	–
DFMO [19]	1	20	35
	0.5	21	
	0.1	32	
	0.05	41	
	0.01	75	
Hydrazinoornithine [24]	1	0	40
	0.1	17	
	0.01	99	
1,4-Diaminobutan-2-one [25]	1	23	25
	0.1	30	
	0.01	63	
α-Methylornithine [26]	1	28	300
	0.1	72	
Phosphorylethanolamine [9]	1	119	
Phosphorylcholine [9]	1	112	
Canaline [27]	1	4	20
	0.1	15	
	0.01	60	
1-Aminooxy-3-aminopropane [18]	1	3	0.06
	0.1	4	
	0.01	16	
	0.001	29	
	0.0001	43	
trans-1,4-Diaminocyclohexane-1-carboxylic acid [28]	1	42	600
	0.1	81	
DFMA [14]	0.5	61	

inhibitors were DFMO, hydrazinoornithine, 1,4-diaminobutane-2-one and canaline. α-Methylornithine and trans-1,4-diaminocyclohexane-1-carboxylic acid were less potent inhibitors. Phosphorylethanolamine and phosphorylcholine which have been reported to inhibit rat ODC [9] had no inhibitory effect on wheat ODC. The ADC inhibitor DFMA inhibited ODC 39% at a concentration of 0.5 mM. The extract might contain arginase, hydrolyzing DFMA to DFMO [10]. Inhibition could also result from a low specificity of DFMA for ODC or ADC, because even the substrates have a low specificity for these enzymes [11].

The only potent ADC inhibitor was DFMA which reduced enzyme activity to 50% at a concentration of 0.01 mM (Table II). Aminooxyaminopropane was less active. Evernic acid, L-canavanine and NSD 1055 were only marginally active. DFMO did not inhibit ADC, indicating that ADC was measured and not ODC. Primary conversion of ^{14}C-labelled arginine to ornithine and subsequent $^{14}CO_2$ release can thus be excluded.

SAMDC was strongly inhibited by MGBG at 0.1 mM and by aminooxyaminopropane at 1 mM (Table III). Berenil and atabrine were only marginally active at 1 mM.

From the inhibitors tested on spermidine synthase (Table V) cyclohexylamine and aminopropylcadaverine showed good activities at 1 mM. Aminooxyaminopropane most probably inhibited spermidine synthase indirectly by its effect on SAMDC. The other compounds were not effective. Dicyclohexylamine is not inhibitory as already demonstrated [12].

The limited data obtained and the extent of enzyme purification does not enable the inhibitor constants to be calculated exactly or the type of inhibition to be established, nevertheless approximate I_{50}-values are given (Tables II–IV). It is clear that several compounds described to be inhibitors of animal

Table V. Inhibition of spermidine synthase in extracts of *T. vulgare*. 1-Aminooxy-3-aminopropane inhibiting SAMDC was added to show its influence on spermidine synthase in the reaction conditions used (the substrate for spermidine synthase is produced by SAMDC in the same mixture). For each compound tested a reference for synthesis and further tests is given.

Inhibitor	Concentration [mM]	Spermidine synthase activity in % compared to control
Control	–	100
Ethambutanol [29]	1	114
Dicyclohexylamine [30]	1	84
Cyclohexylamine [30]	1	0
Norspermidine [31]	1	115
Aminopropyl-cadaverine [32]	1	13
Aminopropyl-hexandiamine [32]	1	91
Aminobutyl-hexahydropyrimidine [33–35]	1	64
Aminobutyl-hexahydropyrimidine methyl [33–35]	1	85
Aminopropyl-hexahydropyrimidine [33–35]	1	84
Aminopropyl-hexahydropyrimidine methyl [33–35]	1	116
bis(Aminopropyl)-cadaverine [33–35]	1	126
Canavalmine [36]	1	137
1-Aminooxy-3-aminopropane [20]	1	0

and bacterial enzymes involved in polyamide biosynthesis showed also effects on plant enzymes. Standard inhibitors such as DFMO, DFMA, MGBG and cyclohexylamine were especially effective. However, many other compounds described in the literature did not show activity. They could be inactive on plant enzymes.

It is interesting to note that a remarkable difference in the amount of ADC and ODC existed in wheat (Table I). In wheat ADC seems to be more important for the production of putrescine at least in this stage of development. Other authors observed differences in the amount of ODC and ADC in other plants also [4, 5]. An influence of polyamine synthesis inhibitors on polyamine levels is more likely to come from ADC inhibitors. A second generation of ADC inhibitors was recently described [13]. They should be of great value for the study of the role of polyamines in plant physiology.

The effective inhibitors of enzymes involved in polyamine biosynthesis will be used to study further the importance of polyamines in plants. It is of special interest, whether a depletion of polyamines in plants leads to similar growth retarding effects as in bacteria or mammalian cells.

Acknowledgements

We thank H. Schneider for synthesizing compounds not commercially available. We are grateful for valuable discussions with U. Gisi, D. Hess, and D. Whitacre. The very skilful secretarial help of Mrs. I. Friess is gratefully acknowledged.

[1] T. A. Smith, Annu. Rev. Plant Physiol. **36,** 117–143 (1985).
[2] A. E. Pegg, Biochem. J. **234,** 249–262 (1986).
[3] A. S. Tyms, J. Antimicrob. Chemother. **17,** 131–135 (1986).
[4] H. Birecka, A. J. Bitonti, and P. P. McCann, Plant Physiol. **79,** 509–514 (1985).
[5] H. Birecka, A. J. Bitonti, and P. P. McCann, Plant Physiol. **79,** 515–519 (1985).
[6] M. R. Suresh and P. R. Adiga, Eur. J. Biochem. **79,** 511–518 (1977).
[7] T. O. Eloranta and E. O. Kajander, Biochem. J. **224,** 137–144 (1984).
[8] B. Yamanoha and S. S. Cohen, Plant Physiol. **78,** 784–790 (1985).
[9] G. M. Gilad and V. H. Gilad, Biochem. Biophys. Res. Commun. **122,** 277–282 (1984).
[10] R. D. Slocum and A. W. Galston, Plant Cell Physiol. **26,** 1519–1526 (1985).
[11] G. L. Prasad and P. R. Adiga, J. Biosci. **10,** 203–213 (1986).
[12] K. W. Batchelor, R. A. Smith, and N. S. Watson, Biochem. J. **233,** 307–308 (1985).
[13] A. J. Bitonti, P. J. Casara, P. P. McCann, and P. Bey, Biochem. J. **242,** 69–74 (1987).
[14] A. Kallio, P. P. McCann, and P. Bey, Biochemistry **20,** 3163–3166 (1981).
[15] M. E. Legaz and C. Vicente, Plant Physiol. **71,** 300–302 (1983).
[16] G. A. Rosenthal, Quart. Rev. Biol. **52,** 155–178 (1977).
[17] T. A. Smith, Methods Enzymol. **94,** 176–180 (1983).
[18] R. M. Khomutov, T. Hyvönen, E. Karvonen, L. Kauppinen, T. Paalanen, L. Paulin, T. Eloranta, R.-L. Pajula, L. C. Andersson, and H. Pösö, Biochem. Biophys. Res. Commun. **130,** 596–602 (1985).
[19] B. W. Metcalf, P. Bey, C. Danzin, M. J. Jung, P. Casara, and J. P. Vevert, J. Amer. Chem. Soc. **100,** 2551–2553 (1978).
[20] H. G. Williams-Ashman and A. Schenone, Biochem. Biophys. Res. Commun. **46,** 288–295 (1972).
[21] R. M. Khomutov, L. L. Zavalova, V. I. Siirku, E. Y. Artamonova, and A. R. Khomutov, Bioorg. Khim. **9,** 130–131 (1983).
[22] E. Karvonen, L. Kauppinen, T. Partanen, and H. Pösö, Biochem. J. **231,** 165–169 (1985).
[23] M. Silverman and E. A. Evans, J. Biol. Chem. **154,** 521–534 (1944).
[24] S. I. Harik and S. H. Snyder, Biochim. Biophys. Acta **327,** 501–509 (1973).
[25] L. Stevens, I. M. McKinnon, and M. Winther, FEBS Lett. **75,** 180–182 (1977).
[26] M. M. Abdel-Monem, N. E. Newton, and C. E. Weeks, J. Med. Chem. **17,** 447–451 (1974).
[27] N. Bagni, P. Torrigiani, and P. Barbieri, Med. Biol. **59,** 403–409 (1981).
[28] P. Bey, C. Danzin, V. Van Dorsselaer, P. Mamont, M. Jung, and C. Tardif, J. Med. Chem. **21,** 50–55 (1978).
[29] U. Bachrach, S. Abzug, and A. Bekierkunst, Biochim. Biophys. Acta **134,** 174–181 (1967).
[30] H. Hibasami and H. Ito, Gann **72,** 512–516 (1981).
[31] J. Massé, J.-C. Laberche, and G. Jeanty, C. R. Acad. Sc. Paris **301** (série III), 27–32 (1985).
[32] C. W. Porter and J. R. Sufrin, Anticancer Res. **6,** 525–542 (1986).
[33] B. T. Golding and I. K. Nassereddin, J. Chem. Soc. Perkin Trans. I, **1985,** 2011–2015.
[34] B. T. Golding and I. K. Nassereddin, J. Chem. Soc. Perkin Trans. I, **1985,** 2017–2024.
[35] B. T. Golding, I. K. Nassereddin, and D. C. Billington, J. Chem. Soc. Perkin Trans. I, **1985,** 2007–2010.
[36] S. Fujihara, T. Nakashima, and Y. Kurogochi, Biochim. Biophys. Acta **805,** 277–284 (1984).