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Assays for the activities of polyamine biosynthetic enzymes using intact tissues

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Abstract — Traditionally, most enzyme assays utilize homogenized cell extracts with or without dialysis. Homogenization and centrifugation of large numbers of samples for screening of mutants and transgenic cell lines is quite cumbersome and generally requires sufficiently large amounts (hundreds of milligrams) of tissue. However, in situations where the tissue is available in small quantities, or one needs to study changes in enzyme activities during development (e.g. somatic embryogenesis), it is desirable to have rapid and reproducible assay methods that utilize only a few milligrams of tissue and can be conducted without homogenization. Here, we report a procedure for the measurement of enzyme activities of the three key decarboxylases involved in polyamine biosynthesis utilizing small quantities of plant tissue without the homogenization and centrifugation steps. Suspension cultures of red spruce (*Picea rubens* (Sarg.)), hybrid poplar (*Populus nigra* × *maximowiczii*), and wild carrot (*Daucus carota*) were used directly to measure decarboxylation of ornithine, arginine and S-adenosylmethionine. Our results demonstrate that this procedure can be used to quantify the activities of arginine decarboxylase (EC 4.1.1.19), ornithine decarboxylase (EC 4.1.1.17) and S-adenosylmethionine decarboxylase (EC 4.1.1.50) in a manner quite comparable to the traditional assays for these enzymes that involve laborious steps of homogenization and centrifugation. © Elsevier, Paris

ADC / carrot / enzyme assays / ODC / polyamines / SAMDC / trees

ADC, arginine decarboxylase / DFMA, DL α-difluoromethylarginine / DFMO, DL α-difluoromethylornithine / EDTA, Na_2-ethylenediamine-tetraacetate / MGBG, methylglyoxal bis(guanylhydrazone) / ODC, ornithine decarboxylase / SAMDC, S-adenosylmethionine decarboxylase

1. INTRODUCTION

Polyamines are naturally occurring aliphatic amines found in all organisms. The most common polyamines in plants are spermidine, spermine and their precursor putrescine. In addition to the much studied roles of polyamines in the regulation of cell division and morphogenesis in plants, they are known to affect the patterns of RNA and protein synthesis, membrane stability, and stress responses of plants. Polyamine metabolism has also been implicated in regulating the metabolism of ethylene and affecting nitrogen pools in plant cells [5, 6, 10, 15].

Polyamine biosynthetic pathways in plants have been well elucidated and are similar to those in animals and microorganisms [6, 12]. In plants, putrescine is formed either by direct decarboxylation of L-ornithine by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17), or by decarboxylation of arginine by arginine decarboxylase (ADC, EC 4.1.1.19) via agmatine and N-carbamoylputrescine intermediates. The distribution of these two enzymes in different plant species is regulated in a developmental and tissue specific manner [13]. Spermidine and spermine are synthesized by the sequential addition of an aminopropyl group to putrescine by spermidine synthase and spermine synthase, respectively. The aminopropyl group is donated by decarboxylated S-adenosylmethionine (SAM), which is produced by S-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50). The decarboxylation of SAM is irreversible, committing SAM exclusively to its use in the

polyamine biosynthetic pathway. The three decarboxylases are believed to be the key regulatory enzymes for polyamine biosynthesis. Specific inhibitors for each of these enzymes have been used in many tissues to manipulate cellular polyamine metabolism. DFMO is a highly effective inhibitor of all animal ODCs while its effectiveness for plant ODCs is quite variable. DFMA and MGBG are generally quite effective in inhibiting the activities of ADC and SAMDC, respectively, in most plant tissues. Considerable attention has recently been focused on isolating mutants defective in the genes for these enzymes [16], cloning and characterization of the genes for these enzymes [15], and genetic manipulation of plant cells using genes coding for these enzymes [5]. In these studies, as in many others involving polyamine metabolism, a need for optimization of rapid and reliable methods for analysis of enzyme activities of these three decarboxylases using small quantities of tissue is obvious [16].

Traditional assays for determination of the activities of ADC, ODC and SAMDC involve trapping $^{14}CO_2$ released from the decarboxylation of [^{14}C]-arginine, [^{14}C]-ornithine and [^{14}C]-SAM, respectively, by tissue homogenates [2]. $^{14}CO_2$ is adsorbed onto filter papers soaked with an alkaline solution. While these methods are quite sensitive and quantitative, they are neither suitable for screening large numbers of tissue samples nor for quantitative analysis of enzyme activities in extremely small (5–10 mg FW) amounts of tissue because of the laborious steps of homogenization and centrifugation. Watson et al. [16] have recently reported a screening procedure for ADC activity in young *Arabidopsis* seedlings. The $^{14}CO_2$ released from [U-^{14}C]-arginine was trapped on filter papers soaked with $Ba(OH)_2$, followed by analysis of trapped radioactivity by exposure of the filter paper to X-ray film. The assay procedure employed live tissue in 96-well plates. The assay yielded semiquantitative comparison of ADC activities among different plants/tissues without resorting to homogenization. However, this technique was not as sensitive as the scintillation counting of radioactivity.

We report here a significant refinement of the enzyme assays for all three key decarboxylases involved in polyamine biosynthesis in plants using intact cells. Our results demonstrate that the procedure can be used to quantify decarboxylation rates of arginine, ornithine and SAM in a manner quite comparable to the traditional assays for these enzymes but without the laborious steps of homogenization and centrifugation, and with relatively small amounts of live tissue. General kinetics of decarboxylation, such as linearity with time of incubation, effects of substrate concentration, and the effectiveness of inhibitors are quite comparable for the two types of assays.

2. RESULTS AND DISCUSSION

Typical enzyme assays are performed using tissue homogenates under conditions that are optimized for temperature, pH, substrate concentration, volume of homogenate, and the length of time during which a linear increase in enzyme activity is observed. Optimized assay conditions do not usually reflect the cellular environment under which the enzymes are active in the plant. For example, temperatures in the range 37–45 °C and pH range 8.2–8.5 are often used for periods of minutes to hours under saturating substrate concentrations. Enzyme activity is often expressed on the basis of per unit tissue fresh or dry weight or per unit soluble protein. While the results provide a useful measure of the buffer extractable enzyme activity in the tissue at a given time, whether or not it reflects the in situ situation of actual enzyme activity is always debatable. In the present study, decarboxylation activities of live intact tissues and homogenized tissue extracts were assayed under comparable conditions in order to determine if the simple protocols with live tissues could be used for screening different tissues/cell lines showing high and low enzyme activities for the three key enzymes involved in polyamine biosynthesis. All results are presented in terms of enzyme activity per gram fresh weight equivalent of tissue. For normalizing the contribution of tissue to the volume of extract or the assay mixture, a factor of 1 mL per gram tissue fresh weight was used.

2.1. Decarboxylation of arginine

Data presented in *table I* show that the rates of decarboxylation of [1-^{14}C]-arginine measured with intact cells were usually higher than those with tissue homogenates for both red spruce and poplar. For carrot cells, however, opposite trends were observed. This could be a reflection of the morphology of the cells which may affect substrate uptake. The red spruce and the poplar cell suspensions consist mostly of single cells or multicellular filaments while the carrot cell suspensions contain compact cell masses. The decarboxylation of arginine was significantly inhibited by DFMA, a suicidal inhibitor of ADC activity. For homogenates, the inhibition was almost 100 % for red

Table I. Comparison of the rates of decarboxylation of [^{14}C]-arginine and the effect of DFMA (DL α-difluoromethylarginine) using intact cells and homogenates for different tissues. Rate of decarboxylation is expressed as (pkat $^{14}CO_2 \cdot g^{-1}$ FW). The data are mean ± SD of two replicates. *, Transgenic cells.

Treatment	Species	Type of tissue	Days of culture	Intact cells	Homogenate
Control	red spruce-A	suspension	4	36.5 ± 4.3	33.7 ± 0.3
1 mM DFMA	red spruce-A	suspension	4	8.4 ± 0.9	0.0 ± 0.0
Control	red spruce-A	callus	11	1.6 ± 0.0	1.7 ± 0.4
1 mM DFMA	red spruce-A	callus	11	1.1 ± 0.3	0.0 ± 0.0
Control	poplar-6A*	suspension	5	21.6 ± 0.6	13.8 ± 0.0
1 mM DFMA	poplar-6A*	suspension	5	5.5 ± 0.2	3.4 ± 0.1
Control	carrot	suspension	4	7.5 ± 0.2	31.2 ± 0.6
1 mM DFMA	carrot	suspension	4	2.6 ± 0.2	13.2 ± 0.1

spruce and 60–70 % for poplar and carrot. In the case of intact cells, about 75 % inhibition was observed with cell suspensions and only 35 % for callus clumps, due possibly to its dependence upon uptake of the inhibitor.

Using red spruce cell suspensions, we studied the effects of the time of incubation, varying substrate concentrations, and pH on the rates of decarboxylation of [1-^{14}C]-arginine by tissue homogenates as well as intact cells. Data in *figure 1* show that the rates of decarboxylation were linear up to at least 90 min in both cases; the rate continued to rise up to 120 min for intact cells but decreased for the homogenates beyond this time. The decrease in enzyme activity beyond 90 min in the homogenates could be due to the proteolytic breakdown of the enzyme. The rates of decarboxylation on fresh weight basis were quite comparable at any given time for the two types of assays. The effect of increasing substrate concentration on decarboxylation showed a typical curve that approached saturation above 2 mM arginine (*figure 2*). The two types of decarboxylation assays showed parallel changes in reaction rates with increasing substrate concentrations, although intact cells always showed somewhat higher activity. Whereas a typical pH response curve with an optimum around 8.4 was observed for the homogenate, no such effect of pH was observed for intact cells (data not shown). This is presumably due to the fact that the external pH often does not significantly change the cellular pH in intact cells [9], thus enzyme activity remains unaffected regardless of the pH of the buffer.

2.2. Decarboxylation of ornithine

The data presented in *table II* show that cell homogenates of all three species contain very little ODC activity. This is consistent with the published results with red spruce and carrot [1, 4, 8, 13]. Even when no ODC activity was detected in assays using homogenates, a small amount of activity was observed using intact cells. This may either reflect actual ODC activity in the cells or it may represent a metabolic breakdown of ornithine to release $^{14}CO_2$ through an alternate pathway. When we used transgenic cell lines of carrot and poplar expressing a mouse ODC cDNA, significantly higher (as compared to non-transgenic cells) rates of decarboxylation of ornithine were observed for both cell types in homogenized extracts as well as in the intact cells. Once again, the rates of decarboxylation in the two types of assays were comparable for poplar, but for carrot, the homogenate yielded higher

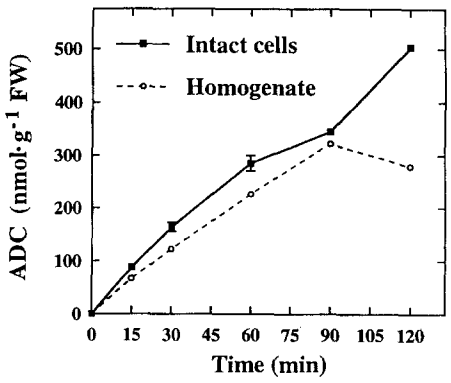

Figure 1. The effect of incubation time on the rate of decarboxylation of [^{14}C]-arginine (ADC activity) in 3-d-old suspension cultures of red spruce line B. For homogenates, the reaction mixture of 300 µL contained 200 µL of cell extract (500 mg cells·mL^{-1} buffer), 50 µL buffer, and 50 µL 12 mM arginine containing 3.7 kBq DL-[1-^{14}C]-arginine. For assays with intact cells, 100 mg washed cells were placed directly in the test tube and to this, 300 µL reaction mixture containing 250 µL extraction buffer and 50 µL arginine as described above was added. The data are mean ± SD of two replicates. The SD bars where not visible are smaller than the size of the symbol.

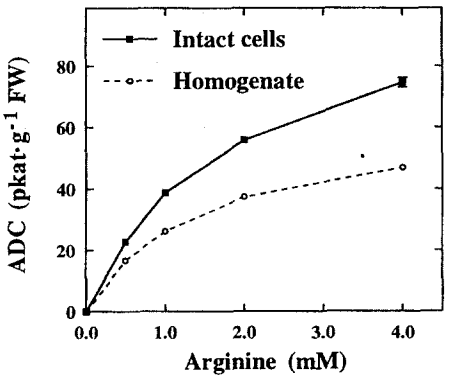

Figure 2. The effect of substrate concentration on the rate of decarboxylation of [^{14}C]-arginine (ADC activity) in 3-d-old suspension cultures of red spruce line B. For homogenates, the reaction mixture of 300 µL contained 200 µL of cell extract (500 mg cells·mL^{-1} buffer), 50 µL buffer and 50 µL proper concentration of arginine containing 3.7 kBq DL-[1-^{14}C]-arginine. Data were adjusted for the ratio of cold to hot substrate. For assays with intact cells, 100 mg washed cells were placed directly in the test tube and to this, 300 µL reaction mixture containing 250 µL extraction buffer and 50 µL arginine as described above was added. The data are mean ± SD of two replicates. The SD bars where not visible are smaller than the size of symbol.

decarboxylation rates than the intact cells with a few exceptions.

It is also known that plant ODC is often not inhibited by DFMO, a potent irreversible inhibitor of animal ODC [12, 13]. The data presented in *table II* are consistent with published results. While native plant ODC, when detected in red spruce cells or carrot leaves, was not inhibited by DFMO, the mouse ODC activity in the transgenic cells was completely inhibited in both the homogenate and the intact cells.

The rate of decarboxylation was linear with time up to 90 min in homogenates, and fell substantially thereafter; it was linear up to at least 120 min with intact cells (data not shown). As with ADC, this decrease in enzyme activity in the homogenate could be due to the degradation of ODC by proteases with time. With respect to substrate concentration, a saturation level was reached beyond 0.5 mM ornithine in homogenates while a small increase in decarboxylation continued up to 4 mM ornithine when intact cells were used (data not shown). This may be due to the limited uptake of ornithine by the intact tissue which may not allow saturating substrate levels to be reached inside the cell. When decarboxylation rates with intact cells were compared at 25 and 37 °C, the rates at 25 °C were only about 30 % of those at 37 °C (data not presented). With the exception of carrot, at 37 °C, the rates of decarboxylation by homogenate and intact cells were quite comparable.

2.3. Decarboxylation of S-adenosylmethionine

In the case of SAM, the rates of decarboxylation were slightly higher for the homogenate than the intact cells except for carrot cells (*table III*). As expected, decarboxylation of SAM was significantly inhibited by 0.2 and 0.4 mM MGBG. The lower rates of decarboxylation of SAM for assays with intact cells are most likely due to the slow uptake of SAM, which may prevent saturation levels of the substrate from being reached in the cells. Nevertheless, the fact that the SAM decarboxylation was almost entirely inhibited by MGBG indicates that this procedure can be used with confidence to follow changes in enzyme activity in small amounts of intact tissue without homogenization.

Table II. Comparison of the rates of decarboxylation of [^{14}C]-ornithine and the effect of DFMO (DL α-difluoromethylornithine) using intact cells and homogenates for different tissues. Rate of decarboxylation is expressed as (pkat $^{14}CO_2 \cdot g^{-1}$ FW). The data are mean ± SD of two replicates. NA, Not applicable; *, transgenic cells.

Treatment	Species	Type of tissue	Days of culture	Intact cells	Homogenate	Enzyme
Control	red spruce-B	suspension	5	1.4 ± 0.0	0.0 ± 0.0	Native ODC
2 mM DFMO	red spruce-B	suspension	5	1.9 ± 0.2	0.0 ± 0.0	Native ODC
Control	poplar	suspension	3	2.0 ± 0.4	0.2 ± 0.0	Native ODC
Control	poplar-6A*	suspension	5	21.4 ± 2.1	18.7 ± 0.4	Mouse ODC
2 mM DFMO	poplar-6A*	suspension	5	0.7 ± 0.1	0.0 ± 0.0	Mouse ODC
Control	carrot-N14*	suspension	5	8.0 ± 0.7	23.8 ± 0.5	Mouse ODC
2 mM DFMO	carrot-N14*	suspension	5	0.9 ± 0.0	0.1 ± 0.0	Mouse ODC
Control	carrot	leaf	NA	4.0 ± 0.4	4.2 ± 0.1	Native ODC
2 mM DFMO	carrot	leaf	NA	4.0 ± 0.2	3.6 ± 0.2	Native ODC

Table III. Comparison of the rates of decarboxylation of [^{14}C]-SAM and the effect of MGBG (methylglyoxal bis(guanylhydrazone)) using intact cells and homogenates for different tissues. Rate of decarboxylation is expressed as (pkat $^{14}CO_2 \cdot g^{-1}$ FW). The data are mean ± SD of two replicates. *, Transgenic cells.

Treatment	Species	Type of tissue	Days of culture	Intact cells	Homogenate
Control	poplar	suspension	4	0.61 ± 0.10	0.99 ± 0.08
0.2 mM MGBG	poplar	suspension	4	0.02 ± 0.01	0.00 ± 0.00
0.4 mM MGBG	poplar	suspension	4	0.03 ± 0.01	0.00 ± 0.00
Control	poplar-6A*	suspension	4	0.85 ± 0.05	1.43 ± 0.14
0.2 mM MGBG	poplar-6A*	suspension	4	0.03 ± 0.00	0.00 ± 0.00
0.4 mM MGBG	poplar-6A*	suspension	4	0.04 ± 0.02	0.00 ± 0.00
Control	red spruce-A	suspension	5	1.92 ± 0.21	2.40 ± 0.20
0.2 mM MGBG	red spruce-A	suspension	5	0.16 ± 0.00	0.14 ± 0.01
0.4 mM MGBG	red spruce-A	suspension	5	0.09 ± 0.03	0.11 ± 0.00
Control	carrot N-14*	suspension	4	2.00 ± 0.17	1.81 ± 0.07
0.2 mM MGBG	carrot N-14*	suspension	4	0.29 ± 0.04	0.31 ± 0.03
0.4 mM MGBG	carrot N-14*	suspension	4	0.29 ± 0.02	0.28 ± 0.02

2.4. Error factors for decarboxylation rates with homogenate vs. intact cells

As described in the introduction, the enzyme assays for the three decarboxylases are usually carried out using tissue extracts at optimized pH, substrate concentration and temperatures. The degree of homogenization of the tissue varies with the tissue type, texture, method of homogenization, and the relative amounts of tissue and buffer. The measurement of enzyme activity is often affected by the action of proteases in the homogenates, non-specific reactions in the homogenates, and the amounts of endogenous substrate and inhibitors. The measurements reflect total enzyme activity without consideration for subcellular compartmentalization of the enzyme or cytoplasmic pH. While some of these parameters can be standardized (e.g. the degree of homogenization) and others can be dealt with through treatments such as dialysis or ammonium sulfate precipitation, the enzyme assays are still subject to significant errors due to other factors. A major advantage of assays with homogenate is that the enzymes are solubilized and are homogeneously mixed with the substrate(s) in the assay tube. Also, since the assays are run at optimized pH, the measurements provide the total amount of enzyme activity in the tissue at the time of analysis.

The decarboxylation assay procedure described here may also suffer from a number of equally serious problems, the most important of which are the rate of uptake of the substrate(s) and the compartmentalization of the enzyme. Assays with intact cells are not subject to the effects of buffer pH, because the external pH often does not significantly affect the cellular pH [9]; thus, the enzyme activity is always being measured at cellular pH. The data presented here demonstrate the consistency and the reliability of results with intact cells with an additional advantage of being able to handle large numbers of small quantities of tissue samples for screening of mutants with elevated or suppressed decarboxylase activities and for transgenic cell lines overexpressing a transgene for these decarboxylases. Furthermore, the procedure can also be used to follow relative changes in decarboxylase activity in the developing tissues such as somatic embryos (that are generally available in small quantities) or cultures subjected to different treatments. We have successfully used this technique to measure ODC and ADC activities in 5–10 mg tissue samples using developing somatic embryos of red spruce (data presented elsewhere).

3. CONCLUSION

The sensitivity, the quantitative nature and the ease with which large numbers of tissue samples can be handled, to assay the activities of these carboxylases by the method described here, should make this method quite useful for analysis of these key enzymes involved in polyamine biosynthesis in large numbers of plant (and possibly animal) tissues. Some situations where this intact cell procedure may provide unique applications include: (a) screening of mutant populations of plants for low or high enzyme activities; (b) following changes in enzyme activities during the development of somatic embryos in cell/tissue cultures using 4–5 mg tissue (only a few embryos); (c) screening of transgenic cell lines overexpressing heterolo-

gous genes for these decarboxylases; and (d) the response of cells to continuous treatment with growth regulators and other chemicals in a flow-through setup. The data presented here demonstrate clearly that the activities of enzymes such as ADC, ODC and SAMDC can be reliably measured using intact cells and tissues instead of homogenates.

4. METHODS

4.1. Cell cultures

Suspension cultures of red spruce (*Picea rubens* (Sarg.)), hybrid poplar (*Populus nigra* × *maximowiczii*) and wild carrot (*Daucus carota*) were used in this study. In the case of poplar and carrot, a control and a transgenic cell line expressing a mouse ODC cDNA was used. The growth conditions for these cultures are described elsewhere: red spruce line A [7], red spruce line B [8], carrot [1] and control cell line of hybrid poplar (*Populus nigra* × *maximowiczii*) [14]. A transgenic cell line of hybrid poplar produced by biolistic bombardment with a plasmid containing mouse ODC cDNA under the control of a duplicated 35S CaMV promoter was characterized through PCR, polyamine analyses, and mouse ODC activity assayed with homogenates (B. Glasheen, S. Bains, R. Minocha, C. Walter and S. Minocha, unpubl. data). In the case of carrot, excised leaf tissue was also used for native ODC assays, since non-transformed control carrot cell cultures do not show ODC activity [13]. Each experiment was repeated two to five times and each treatment was run in duplicate.

4.2. Preparation of homogenates

Cells were collected on Miracloth by vacuum filtration and washed twice with excess deionized distilled water. The pooled cell mass was subdivided into two fractions: one used for decarboxylation assays with homogenates and the other with intact cells. For assays with homogenates, 500 mg cells were homogenized in 1 mL extraction buffer using a Polytron homogenizer (Brinkmann, Littau, Switzerland) for 90 s at 20 000 rpm. The extraction buffer for ODC and ADC contained 50 mM Tris-HCl (pH 8.4), 0.5 mM pyridoxal-5-phosphate, 0.1 mM EDTA and 5.0 mM dithiothreitol (DTT). For the transformed carrot and poplar cell lines expressing the mouse ODC cDNA, the buffer pH was adjusted to 6.8, which is the optimum pH for mouse ODC [3]. For SAMDC, the extraction buffer contained 100 mM potassium phosphate (pH 7.5), 3 mM putrescine and 1 mM DTT. The homogenates were centrifuged at $18\,000 \times g$ for 20 min at 4 °C and the supernatants used for enzyme assays.

4.3. ODC and ADC activity

The activities of ODC (ornithine decarboxylase, EC 4.1.1.17) and ADC (arginine decarboxylase, EC 4.1.1.19) were assayed according to the procedure of Robie and Minocha [13]. The reaction mixture of 300 µL contained 200 µL cell extract, 50 µL buffer and 50 µL 12 mM ornithine containing 1.85 kBq L-[1-^{14}C]-ornithine (New England Nuclear, Boston, MA; sp. act. 2.15 GBq·mmol^{-1}) or 12 mM arginine containing 3.7 kBq of DL-[1-^{14}C]-arginine (Moravek Biochemicals Inc., Berea, CA; sp. act. 2.07 GBq·mmol^{-1}), respectively. For assays involving inhibitors, 20 µL of either water or inhibitor (32 mM DFMO for ODC and 16 mM DFMA for ADC) were added to the reaction mixture. Reaction was run in a 16 × 100 mm Kimble test tube fitted with a rubber stopper holding a polyethylene well (Kontes Scientific Instruments, Vineland, NJ). A 2-cm^2 piece of Whatman 3 MM filter paper soaked with 50 µL Tissue Solubilizer (NCS, New England Nuclear, Boston, MA) was placed in each well to trap liberated $^{14}CO_2$. After incubation at 37 °C for 60 min, 500 µL 0.5 N H_2SO_4 was injected through the stopper to terminate the reaction. After an additional incubation for 30 min, the filter papers were removed and counted for radioactivity. Blanks contained the extraction buffer instead of cell extract. The specific activity of the enzymes is expressed as pkat $^{14}CO_2$ released·g^{-1} tissue FW.

4.4. SAMDC activity

S-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50) was assayed according to Minocha et al. [11]. Briefly, the reaction mixture of 300 µL contained 200 µL cell extract, 50 µL 1.2 mM SAM containing 3.7 kBq of L-[1-^{14}C]-SAM (New England Nuclear, Boston, MA; sp. act. 2.18 GBq·mmol^{-1}), and 50 µL of either water or 1.2 or 2.4 mM MGBG. The collection and counting of $^{14}CO_2$ were done as described above for ODC and ADC, except that the reaction time was 30 min instead of 60 min.

4.5. Assays with intact cells

For decarboxylation assays with intact cells, 100 mg washed cells were placed in the Kimble test tubes directly. The reaction mixture of 300 µL contained

250 µL extraction buffer and 50 µL ornithine, arginine or SAM as described above for assays with homogenates. For inhibitor effects, 20 µL of either water or the inhibitor solution were added to these tubes. The remainder of the procedure was the same as described above for assays with homogenates.

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