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Polyamine metabolism during the growth cycle of tobacco BY-2 cells

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Abstract

We studied polyamine (PA) biosynthesis, oxidation and conjugation in asynchronously dividing cells of tobacco BY-2 cell suspension culture (*Nicotiana tabacum* L.) during 7-day growth cycle. We analyzed the levels of free and conjugated PAs and the activities of biosynthetic and catabolic enzymes during the subculture interval. The contents of free spermidine and spermine started to increase after the inoculation into the fresh medium, positively correlated with the mitotic activity of BY-2 cells and reached their maxima at the beginning of exponential phase on day 3. On the contrary, the endogenous level of free Put showed a transient decline in the lag-phase, and then increased till the end of exponential phase (day 5). The time–course of the content of PCA-soluble conjugates showed a trend similar to that of the free PAs. The inoculation of BY-2 cells into the fresh medium resulted in a sharp increase in the activities of ornithine decarboxylase (ODC, EC 4.1.1.17) and *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50). Arginine decarboxylase (ADC; EC 4.1.1.19) activity remained low during the whole subculture interval. The rise of diamine oxidase (DAO; EC 1.4.3.6) in the first day after subculture coincided with the decrease in free Put level. De novo synthesis of PAs in BY-2 cells after inoculation into the fresh medium and the participation of both PA conjugation with hydroxycinnamic acids and Put oxidative degradation in maintaining of free PA levels during the growth cycle are discussed.

Keywords: ADC; Cell division; DAO; ODC; PAL; Polyamines; SAMDC; Tobacco

1. Introduction

Polyamines (PAs), low molecular mass polycations, are ubiquitous cell components essential for normal growth of both prokaryotic and eukaryotic cells [15]. In higher plants the most prevalent PAs are putrescine (Put), spermidine (Spd) and spermine (Spm). PAs have been shown to affect different growth and developmental processes such as cell division, regulation of morphogenesis, embryogenesis and response to environmental stresses (reviewed in [5,14]). The necessity of tight control of the intracellular free PA level is the same as in the case of plant phytohormones. Apart from the rate of their biosynthesis, the intracellular concentrations of free PAs could be maintained by conjugation, PA degradation through oxidative deamination and depends also on mechanisms of their transport [2].

Two main pathways for the biosynthesis of PAs occur in higher plants. The diamine Put may be formed directly from ornithine via ornithine decarboxylase (ODC; EC 4.1.1.17) or indirectly from arginine via arginine decarboxylase (ADC; EC 4.1.1.19). The existence of two alternative routes for the synthesis of Put could be explained by the different roles of the two enzymes and their specific regulation during the growth and development [16]. It has been suggested that ODC is involved in the regulation of cell division in actively growing plant cell tissue, whereas ADC activity is generally linked to stress responses and morphogenetic processes [11,16,21]. *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) is essential for Spd and Spm biosythesis. Spd is formed from Put and Spm from Spd by successive addition of aminopropyl groups derived from decarboxylated *S*-adenosylmethionine that is generated by the activity of SAMDC.

Abbreviations: ADC, arginine decarboxylase; DAO, diamine oxidase; EDTA, ethylenediaminetetraacetic acid; GABA, γ-aminobutyric acid; NA, nucleic acid; ODC, ornithine decarboxylase; PA, polyamine; PAL, phenylalanine ammonia-lyase; PAO, polyamine oxidase; PCA, perchloric acid; Put, putrescine; SAMDC, *S*-adenosylmethionine decarboxylase; Spd, spermidine; Spm, spermine.

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In plant cells, PAs occur as free molecular bases and covalently linked to small molecules, especially hydroxycinnamic acids (soluble conjugated PAs), as well as to high molecular-mass substances like hemicelluloses and lignin and in small amounts also to proteins (insoluble conjugated PAs). The physiological function of PA conjugates is still under discussion. It has been proposed that PA conjugates could act as storage forms, as a means of transport and/or they could be important in the regulation of the free PA forms (reviewed in, [18]).

The studies with mammalian cells showed that direct binding of PAs to nucleic acids (NA) and their ability to modulate NA conformation and NA-protein interaction represent an important molecular mechanism of PA action in cell proliferation [37]. The relationship between increased contents of PAs and cell division has been demonstrated in plants by numerous authors (e.g. [10,27]). The direct correlation between PA level and DNA synthesis was found in *Helianthus tuberosus* and in sugar beet cells [10,39]. Moreover, the increase in ODC activity was observed prior to the onset of the cell division [17,36]. Therefore, it is proposed that an increase in PA biosynthesis and an increase in PA levels belong to the first events following stimulation to cell division [10,28].

PA catabolism has been studied especially due to its intermediates (mainly H_2O_2) that play important roles in the growth and development of plants as well as in the plant response to various forms of stresses [2]. Two catabolic enzymes were found in plants. Diamine oxidase (DAO; EC 1.4.3.6), which primarily catalyses the conversion of Put to ammonia, H_2O_2 and pyrroline and polyamine oxidase (PAO; EC 1.5.3.) with high affinity towards Spd and Spm. Although it has been shown that oxidative deamination plays an important role in the regulation of free Put level during cell division [40], catabolism of PAs in relation to the regulation of intracellular PA levels is less studied.

In order to determine how PA metabolism operates in suspension cultures we chose tobacco BY-2 cell suspension culture. BY-2 cells are highly synchronizable and therefore represent a suitable system for studying cell cycle in plants. Alterations in free PA contents throughout the cell cycle of BY-2 cells and their possible participation in the regulation of protein kinase CK2 enzymatic activity was already described in Espunya et al. [9]. In the present work we focused on the role of PA biosynthesis, oxidation and conjugation in maintenance of free intracellular PA levels during the growth cycle. The studies of possible mechanisms of PA homeostasis during BY-2 cell cycle will be the aim of our further work.

2. Results

2.1. Growth of culture

The growth of BY-2 cells followed a sigmoidal curve after inoculation into fresh medium with three common phases:

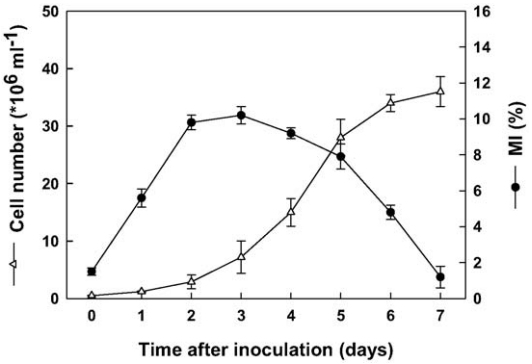

Fig. 1. Changes in cell number and mitotic index in tobacco BY-2 cells during 7-day growth cycle. Each value represents the mean of three independent experiments ± S.E.

lag-phase (days 0–2), exponential phase (days 2–5) with the maximum of mitotic activity and stationary phase (days 5–7). The growth of cell suspension culture was characterized by cell number and mitotic index (Fig. 1).

2.2. Free and conjugated PA contents

Free and conjugated (PCA-soluble and PCA-insoluble conjugates) PA levels were determined each day during 7-day growth cycle of BY-2 cells (Fig. 2A–C). All three PAs were present both in free and conjugated forms. The ratio between these fractions changed during the subculture interval. The marked enhancement in the level of PA conjugates was observed in the exponential phase—on days 3 and 4 (up to 65% of the total PA content) (Fig. 3). The most abundant PA in free and conjugated form was Put and in the lowest concentration occurred Spm. Changes in free PA levels in the cell population are presented in Fig. 2A. The endogenous level of free Put showed a transient decline in the first two days of culture, in spite of marked induction of ODC activity, thereafter increased and reached the maximum value 2 days later than Spd and Spm (on day 5). Free Put content returned to approximately initial level on day 7. The course of the free Spd and Spm levels positively correlated with the changes in mitotic activity showing the maximum on day 3 (Figs. 1 and 2A). The endogenous levels of free Put, Spd and Spm exhibited regular pattern of increase and decline during each subculture cycle.

On Fig. 2B are presented alterations in the levels of PCA-soluble conjugates during the growth cycle and the time–course of the activity of PAL, key enzyme for the biosynthesis of phenylpropanoid conjugation partners for free PAs. Marked stimulation of PAL activity with the peak at 12 h after inoculation was found in BY-2 cells. Following that its activity declined to the basal level on day 3. The time–course analysis of the contents of PCA-soluble conjugates showed a trend similar to that of the free Spd and Spm. The PCA-soluble conjugated forms of the most abundant PA, Put, started to accumulate later, however, their highest content was observed 1 day before the maximum of free Put level occurred.

PCA-insoluble conjugates, the third determined PA fraction, were detected only in small amounts, all three PAs i.e.

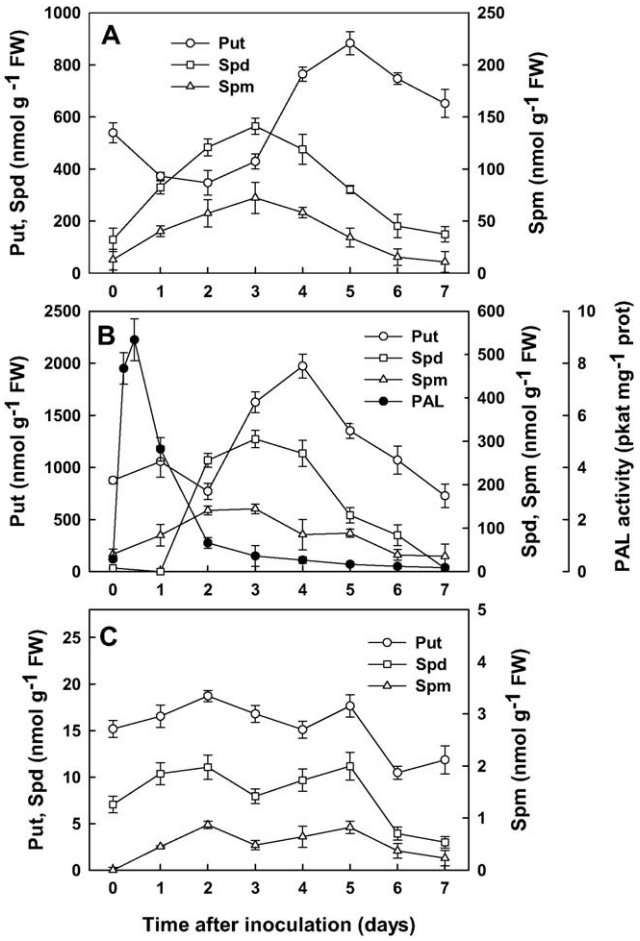

Fig. 2. Changes in PA (Put, Spd and Spm) levels in tobacco BY-2 cells during 7-day growth cycle. (A) Free forms. (B) PCA-soluble conjugates and the time–course of PAL activity. (C) PCA-insoluble conjugates. Each value represents the mean of three independent experiments ± S.E.

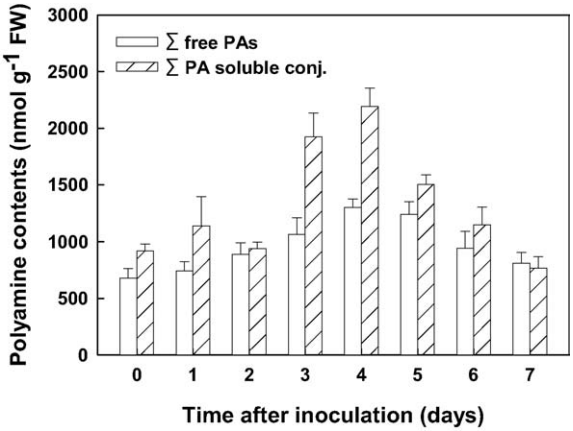

Fig. 3. Changes in free PAs (represented by the sum of their values of free Put, Spd and Spm) and their PCA-soluble conjugates (represented by the sum of their values of PCA-soluble conjugates of Put, Spd and Spm) during 7-day growth cycle. Bars represent S.E. of three replicates.

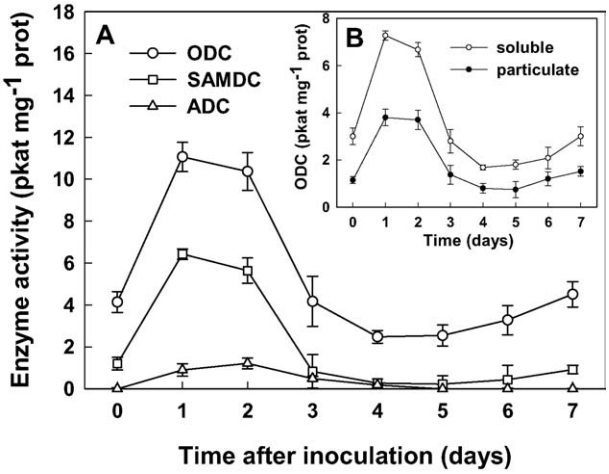

Fig. 4. (A) Time course of PA biosynthetic enzymes, ODC, ADC and SAMDC, in tobacco BY-2 cells during 7-day growth cycle. (B) Time course of ODC activity found in soluble and particulate fractions in tobacco BY-2 cells during 7-day growth cycle. Each value represents the mean of three independent experiments ± S.E.

Put, Spd and Spm showed slight changes with two maxima on days 2 and 5 of BY-2 growth cycle (Fig. 2C). These moderate maxima coincided with the end of lag-phase and with the end of exponential phase of growth cycle.

2.3. Activity of PA biosynthetic enzymes

Biosynthetic enzyme activities were measured both in the soluble and the pellet fractions. However, the only enzyme found in the pellet fraction was ODC (about one third of its whole biosynthetic activity (Fig. 4B)). As seen from the Fig. 4 the subculture of BY-2 cells into the fresh medium caused sharp increase in ODC (values represent the sum of both soluble and pellet bound ODC activities) and SAMDC activities during the lag-phase and thereafter they continually decreased till the end of exponential phase (day 5; Fig. 4). A moderate increase was observed again during the stationary phase of culture. ADC activity remained low during the whole subculture interval with a slight increase in the lag-phase (Fig. 4). In the pellet fraction no ADC activity was found, its activity was probably too low to be detected.

2.4. Activity of diamine oxidase

The rise in the DAO activity on the first day after inoculation correlated with the decrease in free Put level during the lag-phase of BY-2 growth cycle (Fig. 5). The decline of DAO activity in the exponential phase coincided with the increase in free Put levels and with the accumulation of its PCA-soluble conjugates. A slight increase in DAO activity was observed in the stationary phase when the contents of both Put forms decreased (Fig. 5).

3. Discussion

Inoculation of plant suspension cultures into fresh medium results in the activation of cell metabolism. In BY-2 cells, the

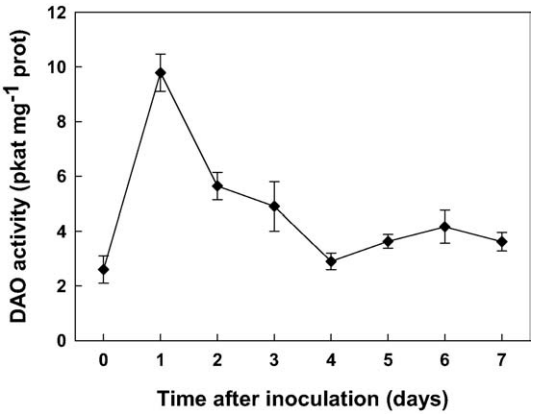

Fig. 5. Time course of DAO activity in tobacco BY-2 cells during 7-day growth cycle. Each value represents the mean of three independent experiments ± S.E.

short lag-phase was followed by the exponential phase during which the cell number sharply increased (Fig. 1). It is well documented in many plant systems that dividing tissues exert high levels of PAs and activities of their biosynthetic enzymes [3,27,39]. The present data show that after the subculture of BY-2 cells the levels of free Spd and Spm positively correlated with the rise of cell number, i.e. with the increase in mitotic activity of cells, and reached their highest values in the onset of the exponential phase (day 3). Whereas the content of free Put, in spite of marked induction of ODC activity, showed the transient decline in the lag-phase and the peak of its content was shifted to the end of exponential phase (Figs. 2A and 4). These data do not coincide with findings obtained by Fowler et al. [10] in sugar beet cell suspension culture. Marked increases in concentration of both Put and Spd were observed in sugar beet cells after stimulation of quiescent cells to the cell division. However, the decline in the free Put level has been demonstrated in *Nicotiana tabacum* and in *Medicago varia* cells during the period of DNA synthesis [26,27]. The decrease in free Put level in the lag-phase could be explained by further utilization of Put for the biosynthesis of Spd and Spm and/or by high DAO activity in this phase of BY-2 growth cycle (Figs. 2A and 5). This is in good agreement with the data concerning stimulated activity of DAO during the S-phase of cell cycle and the simultaneous drop in Put content found in *H. tuberosus*. A direct correlation between the biosynthesis and oxidation of Put was also demonstrated in the period of intense metabolism during tuber formation of *H. tuberosus* [39,40]. These results support the view that catabolic enzymes play an important role in the regulation of free PA levels at least in cells in the stage preceding the onset of cell division. Nevertheless, we should not omit the other DAO important roles in the cell metabolism. As already mentioned above DAO reaction products are pyrroline, H_2O_2 and ammonia. Pyrroline can be further catabolized to γ-aminobutyric acid (GABA) and subsequently transaminated and finally incorporated into the Krebs cycle. This pathway ensures the recycling of carbon and nitrogen [38]. It is further suggested that the DAO-mediated catabolism may act as a way to generate GABA and H_2O_2, both being considered as active secondary messengers [20,31]. There are still some unanswered questions about the precise role of GABA in rooting process. The conversion of Put to GABA might be essential for in vitro root induction in poplar shoots [12], however, this finding was not confirmed in cork oat and grapevine shoots [23]. H_2O_2 together with free radicals represent harmful products of catabolic reaction and for cells their accumulation is toxic and could finally lead to the cell death [29]. This might be a reason for relatively short time of stimulation of DAO activity in BY-2 cells (on days 1 and 2) (Fig. 5). Another explanation for the sharp decline on day 2 could be lower demand of the regulation of free Put level in the actively dividing cells during the exponential phase.

The detected high levels of PCA-soluble conjugates of Put (70–80% of the whole Put pool in the exponential phase; Fig. 2B) seem to be the common phenomena in *Nicotiana* and have been reported by several authors [19,27]. The biological function of conjugated PAs remains still unclear. Our previous results [7,8] confirmed their ability to modulate free PA level. We showed in alfalfa cell suspension cultures and in oak embryogenic cultures that the rate of PA conjugation with hydroxycinnamic acids influenced the endogenous free PA level and, indirectly, cell division. Stimulation of PAL activity in the beginning of lag-phase of BY-2 growth cycle was followed by the accumulation of PCA-soluble PA conjugated forms (Figs. 2B and 3). Soluble conjugates of Spd started to increase after short delay from day 2 (Fig. 2B). The increase in Put conjugated forms coincided with the decline of DAO activity 1 day later (Figs. 2B and 5). The total content of PCA-soluble conjugated forms reached the maximum in the exponential phase (Fig. 3). These results support the suggestion of their involvement in maintaining of free PAs concentrations. PA conjugation seems to be advantageous for the long-term regulation of free PA levels because the risk of accumulation of harmful metabolites, produced by oxidative deamination, is reduced. Relatively low levels of PCA-insoluble conjugates found in BY-2 cells are in agreement with other data presented in plant tissues (Fig. 2C) [7,27], their higher amounts were found in connection with differentiation processes such as flowering [34].

The activities of the key biosynthetic enzymes ODC, ADC and SAMDC showed an increase after inoculation of BY-2 cells into fresh medium (Fig. 4). These results are in concert with the data obtained in plant and animal cells, where the increase of PA biosynthetic enzymes preceded cell division [10,24]. Although the number of studies on PAs is constantly growing, it is still unclear, whether ODC or ADC plays the main role in the Put biosynthesis in plants. Our results imply that in BY-2 cells is ODC the main enzyme involved in Put biosynthesis and that the enzyme is mainly localized in the soluble fraction. Only one third of ODC activity was found in the pellet fraction (Fig. 4B). The data are in correlation with findings in *N. tabacum*, where the soluble fraction of ODC activity was larger than the particulate one [1]. The studies concerning the subcellular localization of ODC and ADC

activities indicated that both proteins are active in cytosolic as well as in particulate fraction [4,30,35]. Nevertheless, it seems that the ratio between these two fractions is dependent on plant species and/or on plant tissue. There was not found any ADC activity in the pellet fraction of BY-2 cells.

As mentioned above, SAMDC activity also reacted with a rapid increase to the subculture of BY-2 cells and after 2 days, in the beginning of exponential phase, declined (Fig. 4). It was previously found out that tobacco SAMDC protein has very short half-life. It is therefore suggested that the repression of SAMDC synthesis by its products, Spd and Spm, represents an important regulatory mechanism [32].

In summary, we have shown that the inoculation of BY-2 cells into fresh medium activated PA metabolism. The maxima of activities of biosynthetic enzymes, ODC and SAMDC, occurred rapidly after the subculture. The contents of free spermidine and spermine positively correlated with the mitotic activity of BY-2 cells and reached their maxima at the beginning of exponential phase of growth. The transient decline in free putrescine level in the lag-phase negatively correlated with the rise of diamine oxidase activity in the first days after subculture. The content of free putrescine then continually increased till the end of exponential phase. Our results led us to conclude that the cellular levels of metabolically active free PAs are regulated via oxidative deamination in the lag-phase and formation of soluble conjugated forms especially during the exponential phase of BY-2 growth cycle.

4. Methods

4.1. Plant material

The tobacco BY-2 cell line (*N. tabacum* L. cv. Bright Yellow 2) was maintained as previously described [22]. The cells were regularly subcultured once a week (inoculation density approximately 5×10^5 cells ml^{-1}) and cultivated at 27 °C in darkness.

The cells were harvested for biochemical studies after 6 and 12 h (PAL activity), and then every day of culture. The samples were stored at –80 °C before analyses.

4.2. PA analysis

The cells were ground in liquid nitrogen and extracted overnight at 4 °C with 5% (v/v) perchloric acid (PCA) (100 mg fresh weight tissue ml^{-1} 5% PCA). 1,7-Diaminoheptane was added as an internal standard. The extracts were centrifuged at 21,000 × *g* for 15 min, and then PCA-soluble free PAs were determined in one-half volume of the supernatant. The remaining supernatant and pellet were acid hydrolyzed in 6 M HCl for 18 h at 110 °C to obtain PCA-soluble and PCA-insoluble conjugates of PAs as described by Slocum et al. [33]. Standards (Sigma-Aldrich, St Louis, MO, USA), PCA-soluble free PAs, and acid hydrolyzed PA conjugates were benzoylated. HPLC analysis of benzoyl-amines was performed on Beckman-Video Liquid Chromatograph equipped with UV detector (detection at 254 nm) and C_{18} Spherisorb 5 ODS2 column (particle size 5 μm, column length 250 × 4.6 mm) according to the method of Slocum et al. [33].

4.3. Ornithine decarboxylase, arginine decarboxylase and S-adenosylmethionine decarboxylase assays

Ornithine decarboxylase (ODC; EC 4.1.1.17), arginine decarboxylase (ADC; EC 4.1.1.19) and *S*-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) were determined by a radiochemical method as described by Tassoni et al. [34]. Samples were extracted in 3 vol. of ice-cold 0.1 M Tris–HCl buffer, pH 8.5, containing 2 mM β-mercaptoethanol, 1 mM EDTA and 0.1 mM pyridoxal phosphate, and centrifuged at 20,000 × *g* for 30 min at 4 °C. Aliquots (0.1 ml) of both supernatant (soluble fraction) and resuspended pellet (particulate fraction) were used to determine ODC and ADC activity. Enzyme activity assays were performed by measuring the $^{14}CO_2$ evolution from 7.4 kBq L-[1-^{14}C]ornithine (1.92 GBq $mmol^{-1}$, Amersham Pharmacia Biotech, UK) or 7.4 kBq L-[U-^{14}C]arginine (11.5 GBq $mmol^{-1}$ Amersham Pharmacia Biotech), for ODC and ADC, respectively, in the presence of 2 mM unlabeled substrate during a 1.5-h incubation at 37 °C. CO_2 was entrapped in hyamine hydroxide and the radioactivity was counted on liquid scintillation analyzer, Tri-Carb 2900TR, Packard.

To determine SAMDC activity samples were homogenized in 3 vol. of 0.1 M phosphate buffer, pH 7.6 containing 2 mM β-mercaptoethanol and 1 mM EDTA, and centrifuged at 20,000 × *g* for 30 min at 4 °C. The supernatant and resuspended pellet (0.1 ml aliquots) were incubated separately with 3.7 kBq [1-^{14}C]*S*-adenosylmethionine (2.15 GBq $mmol^{-1}$, Amersham Pharmacia Biotech) in the presence of 2.8 mM unlabeled substrate and 3 mM putrescine. $^{14}CO_2$ evolution was measured for 1 h at 37 °C. The radioactivity was counted on liquid scintillation analyzer, Tri-Carb 2900TR, Packard.

Protein content was measured according to Bradford's method using bovine serum albumin as a standard [6].

4.4. Diamine oxidase assay

Diamine oxidase (DAO, EC 1.4.3.6) activity was assayed by a spectrophotometric method based on detection of the aldehyde with *cis*-1,4-diamino-2-butene as the substrate [25]. Samples were homogenized in 0.1 M Tris–HCl buffer, pH 8.5, containing 2 mM mercaptoethanol and 1 mM EDTA, and centrifuged at 20,000 × *g* for 15 min at 4 °C. The reaction mixture contained 0.1 M Tris–HCl buffer, pH 8.5, catalase (25 μg) and 0.01 M *cis*-1,4-diamino-2-butene. The reaction was started by addition of 0.2 ml supernatant, incubated for 1 h at 37 °C and stopped by adding 1 ml of Ehrlich's reagent. The reaction mixture was incubated at 50 °C for 5 min, and then chilled on an ice bath before reading the absorbance of produced pyrrol at 563 nm. Enzymatic activity is expressed in pkat mg^{-1} prot.

4.5. Phenylalanine ammonia-lyase assay

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) was extracted and its specific activity determined by the modified method of Jangaard [13]. The amount of cinnamic acid produced in the assay mixture was determined by monitoring the absorbance at 275 nm. Enzymatic activity is expressed in pkat mg^{-1} prot.

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