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Effect of spermidine and its metabolites on the activity of pea seedlings diamine oxidase and the problems of biosensing of biogenic amines with this enzyme

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ABSTRACT

Spermidine is one of the several biogenic amines, produced during the microbial decarboxylation of proteins. Individual biogenic amines in the formed mixtures are frequently analyzed with oxygen sensor based biosensors, as their content serves as a good biomarker for the determination of food quality. In these biosensors, diamine oxidase from pea seedlings (PSAO), catalyzing the oxidation of various biogenic amines by dissolved oxygen is commonly used for the bio-recognition of amines. However, in the presence of spermidine and/or its metabolite 1,3-diaminopropane, the activity of PSAO and the sensitivity of PSAO-based biosensors decrease due to inhibition. The inhibition constant of soluble spermidine, acting as an inhibiting substrate toward PSAO, was found to be (40 ± 15) mM in freshly prepared solution and (0.28 ± 0.05) mM in solution, incubated 30 days at room temperature. The inhibition constant of 1,3-diaminopropane, acting as a competitive inhibitor, was (0.43 ± 0.12) mM as determined through the oxidation reaction of cadaverine. The metabolic half-life of soluble spermidine was 7 days at room temperature and 186 days at 4 °C. The kinetic measurements were carried out with an oxygen sensor; the composition of the solution of degraded spermidine was analyzed with MS.

1. Introduction

Biogenic amines are biologically active low molecular weight compounds, formed by microbial decarboxylation of protein-containing material from the corresponding amino acids or by transamination of aldehydes and ketones, catalyzed by amino acid transaminases (EC 2.6.1.21) [1]. Putrescine (1,4-diaminobutane), cadaverine (1,5-diaminopentane) and spermidine (*N*-(3-aminopropyl)-1,4-butanediamine), also known as biogenic polyamines, are primarily the products of microbial activity [2] and used to serve as natural biomarkers for the determination of food quality [3–7]. The preliminary compounds of the formation of biogenic amines are basic amino acids—arginine, histidine and lysine, and ornithine, formed from arginine in urea cycle. Both arginine and ornithine, which are decarboxylated by arginine decarboxylase (EC 4.1.1.19) and ornithine decarboxylase (EC 4.1.1.17) respectively, are turned into putrescine [8,9], which can be further converted into spermidine. The well-characterized ornithine decarboxylase is specific toward ornithine and does not produce cadaverine from lysine, although ornithine and lysine are structurally very closely related as their decarboxylation products putrescine and cadaverine [10]. Lysine is converted into cadaverine by lysine decarboxylase (EC 4.1.1.18) [10]. Besides polyamines, the most common biogenic amine is histamine, product of histidine decarboxylation (EC 4.1.1.22) [11]. The putrefaction of proteins, macromolecules consisting of the residues of different amino acids, leads to the formation of different mixtures of biogenic amines, depending on the protein content.

Diamine oxidase (EC 1.4.3.22) is a Cu^{2+}-dependent enzyme which catalyzes the oxidative deamination of different amines and is widely used for the bio-recognition of biogenic amines in biosensors. The overall deamination reaction, actually following the ping-pong mechanism [12–14], can be presented as follows:

$$R - CH_2NH_3^+ + O_2 + H_2O \rightarrow NH_4^+ + R - CHO + H_2O_2 \quad (1)$$

The activity of pea seedlings diamine oxidase (PSAO) toward different biogenic amines varies within a wide range [15–18]. Data about the substrate specificity of PSAO, presented by different authors, is variable and depends on the applied experimental method [7]. The sensitivity of PSAO is the highest toward symmetric diamines—cadaverine and putrescine. In addition to these two amines, a noticeable activity has been found toward spermidine;

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Table 1
Substrate specificity of pea seedling diamine oxidase.

Biogenic amine	Specific activity of PSAO (%)	Detection system	Ref.
Cadaverine	100	Electrochemical sensor: enzyme modified graphite electrode	[19]
Putrescine	80		
Spermidine	24		
Histamine	11		
Cadaverine	100	Electrochemical sensor: enzyme modified carbon paste/graphite powder electrode	[20]
Putrescine	96		
Spermidine	17		
Histamine	9		
Cadaverine	100	Fiber optical sensor: enzyme modified magnetic chitosan microparticles	[21]
Putrescine	117		
Spermidine	19		
Histamine	13		
Cadaverine	100	Fiber optical sensor: enzyme modified SEPABEADS® EC-HA 403	[21]
Putrescine	126		
Spermidine	56		
Histamine	17		
Cadaverine	100	Oxygen sensor	[22]
Putrescine	86		
Histamine	10		

the activity toward histamine is much lower [19–22]. A condensed overview of substrate specificity of PSAO is presented in Table 1.

Most biogenic amines are stable, except spermidine, which undergoes quick metabolic changes. Ruiz-Capillas and Moral have studied the changes of biogenic amines concentrations in fish samples during storage in different atmosphere [23]. They noticed that the concentrations of all studied biogenic amines increased, but the concentration of spermidine fluctuated in time. A similar fluctuation has been reported in other studies [24–27]. This phenomenon can be the result of the action of microorganisms [28,29] or the spermidine non-catalytic oxidation [18].

The oxidation of spermidine by molecular oxygen initially yields 1,3-diaminopropane (1,3-DAP) and 4-aminobutyraldehyde; the latter undergoes rapid spontaneous intramolecular Schiff-base formation to yield Δ^1-pyrroline [30]:

$$\text{Spermidine} + \frac{1}{2}O_2 \rightarrow \text{1,3-diaminopropane} + \text{4-aminobutyraldehyde} \underset{\Delta^1 - \text{Pyrroline}}{\downarrow} \quad (2)$$

Δ^1-Pyrroline formation is also reported during the oxidation of putrescine, which similarly to spermidine is initially converted into γ-aminobutyraldehyde further forming Δ^1-pyrroline [31]. The essential difference between spermidine and putrescine oxidation is the formation of 1,3-diaminopropane from spermidine.

It has been shown that diamine oxidase from plants has no catalytic activity toward 1,3-diaminopropane [32]. On the contrary, 1,3-diaminopropane can act as an inhibitor for this enzyme [33–36]. So, the formed 1,3-diaminopropane influences the detection of biogenic amines in real samples, like food and beverages with PSAO-based biosensor.

The aim of the present study was to characterize the potential inhibitory effect of spermidine and its metabolite 1,3-diaminopropane on the activity of pea seedlings diamine oxidase and the biosensing of individual biogenic amines or their total concentration in mixtures, forming during putrefaction of proteins. The catalytic activity of PSAO and the sensitivity of PSAO-based biosensors change in the course of measurements of biogenic amines due to the irreversible deamination of spermidine. We used oxygen sensor to follow the PSAO-catalyzed oxidation reactions; the spermidine decay was additionally analyzed with MS.

2. Materials and methods

2.1. Chemicals and reagents

Spermidine (SPD), 1,3-diaminopropane (1,3-DAP) and cadaverine (CAD) standards were purchased from Sigma–Aldrich (Germany). Diamine oxidase was isolated and purified from pea (*Pisum sativum*) seedlings as described in [32]. PSAO activity in the enzyme extract was 12.0 U/ml. The extract was stored at −20 °C and used within 4 h after melting at 4 °C.

All chemicals were of analytical grade unless otherwise stated. All aqueous solutions were prepared with ultrapure DI water (18.2 MΩ/cm).

2.2. Experimental procedures using biosensing system

The change of dissolved oxygen concentration in reaction medium due to the oxidation of biogenic amines was followed with a Clark-type oxygen sensor (covered with 25 μm thick polyethylene film with the area of 5.65 cm^2, *Elke Sensor* LLC, Estonia). All experiments were carried out under constant stirring in air-saturated 0.1 M phosphate buffer solutions (pH 7.00, in DI water) at 25.0 °C in thermostated air-tight reaction cell. The sensor was put into the mixture of biogenic amines and the reaction was started by injection of PSAO extract; the final PSAO concentration in the reaction medium was always 0.043 U/ml. The sensor signal was registered automatically with 0.5 s interval; the obtained data were normalized and used for the calculation of reaction parameters.

2.3. Calculation of reaction parameters

The reaction characteristic parameters were calculated using the dynamic model for biosensors [37]. This model enables to calculate the reaction kinetic and steady-state parameters from the transient phase response, minimizing the influence of side processes (H_2O_2 degradation, oxygen mass transfer through the liquid–air surface etc.) and avoiding the uncertainty of determining the steady state. The calculated steady state parameter is depending hyperbolically on amine concentration.

2.4. Mass spectrometric measurements

The measurements were carried out using an Agilent XCT ion trap mass spectrometer equipped with electrospray interface (ESI). For infusion experiments, solutions were directly infused into mass spectrometer with syringe pump at flow rate 0.5 ml/h. Spectra was collected for 1 min and averaged. ESI source parameters were: nebulizer gas (nitrogen) pressure 50 psi (345 kPa), drying gas (nitrogen) flow rate 12 l/min and drying gas temperature 350 °C.

3. Results and discussion

The activity of PSAO toward studied amines was characterized with the calculated normalized total signal change parameter *A*, corresponding to the maximum signal change at steady state conditions [38]. The dependence of this parameter on the concentration of spermidine is presented in Fig. 1. This dependence had an irregular bell-shape form with a flat maximum at spermidine

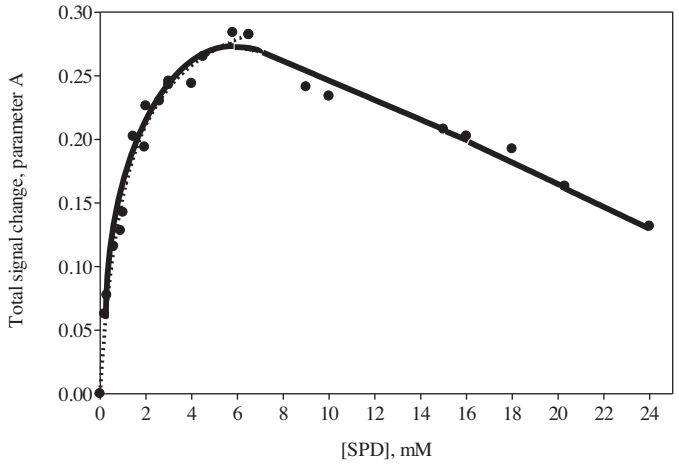

Fig. 1. The total signal change parameter *A* for spermidine. Measurements were carried out in 0.1 M phosphate buffer (pH 7.0) at 25.0 °C, [PSAO] = 0.043 IU/ml. Experimental points are the mean of at least 3 measurements.

concentration around 6 mM, indicating that at higher concentrations spermidine acts as an inhibiting substrate. This mechanism of action is analogs to that considered for uncompetitive inhibition. The value of K'_M, defined as $k_{-1} + k_2/k_1$ (similar to *Michaelis–Menten* constant K_M, but the rate equation of this kind reactions is different from *Michaelis–Menten* equation), was calculated using the biosensor dynamic model [39] from the initial part of the plot and was equal to 0.88 ± 0.34 mM. This value for spermidine as a substrate is similar to the K_M values obtained in earlier studies [40–43]. The value of spermidine inhibition constant K_{si}, calculated as $K_{si} = c^2/K'_M$, where c is the substrate concentration at the maximum of the plot, was (40 ± 15) mM. The theoretical maximum value of the total signal change parameter, A_{max} was 0.34 ± 0.01 mM. The fact that spermidine is an inhibiting substrate to PSAO can be the reason of the fluctuations of spermidine concentration during storage, determined with PSAO-based biosensors [23–26].

We also studied the effect of spermidine on the detection of other biogenic amines in mixed samples with PSAO, using cadaverine as a standard substrate. Cadaverine was chosen because PSAO has the highest selectivity toward this substrate [44,45]. In freshly prepared spermidine solutions, the dependences of the total signal change parameter *A* on spermidine concentration at different cadaverine concentrations are presented in Fig. 2. Along with the experimental results (shown in black), theoretical curves illustrating the summarized effect of spermidine and cadaverine if no additional inhibition by spermidine on cadaverine oxidation occurs are shown in gray.

It can be seen that spermidine acts as an inhibiting substrate also in the mixtures of spermidine and cadaverine. This inhibiting effect is bigger at higher cadaverine concentrations and the plot maximums shift toward lower spermidine concentrations along with the increase of cadaverine concentration. At cadaverine concentration 1.5 mM, exceeding over five times its K_M value (K_M for cadaverine is 0.26 mM [34]), the enzyme is inhibited already at minute spermidine concentrations.

Comparing the experimental results with the theoretical curves we can see that the calculated reaction parameter underestimates the total amine content in multi-substrate systems when spermidine is present. So, in the mixtures with other amines, spermidine acts as an inhibitor to PSAO already at lower concentrations than in its single-substrate solution.

Spermidine is a naturally existing biogenic amine in foods. Spermidine concentrations up to 22 ppm has been found in white fresh fish [23,27]. In fresh fish, the levels of all biogenic amines are low [46,47] and spermidine does not interfere the determination of these compounds with PSAO-based biosensors. During storage, the concentrations of biogenic amines (incl. spermidine) are rising rapidly. In tuna fish, stored at 0–4 °C, the spermidine concentration was 47 ppm [23,27] and stored at room temperature, up to 70 ppm [23]. In addition, PSAO-based biosensor studies have indicated fluctuations of spermidine concentration between 15 and 70 ppm during the storage of food, suggested to be caused also by the degradation of spermidine [23].

To study the effect of spermidine degradation more thoroughly, we followed the spermidine decay in time and determined its inhibiting effect on PSAO-catalyzed cadaverine oxidation (Fig. 3). The degradation of SPD was evaluated using the decrease of the total signal change parameter *A* in time (Fig. 3A). The estimated spermidine half-life at 4 °C was 186 days and at room temperature 7 days.

In the time course, spermidine is oxidized into 1,3-diaminopropane and Δ^1-pyrroline [30]. The effect of spermidine, indicating no detectable substrate activity toward PSAO, was studied toward PSAO-catalyzed cadaverine oxidation at the same cadaverine concentrations (Fig. 3B) as with the freshly prepared spermidine (Fig. 2). It can be seen that spermidine acts only as an inhibitor here. By analyzing the Lineweaver–Burk plots, the inhibition of PSAO can be characterized as competitive and can be caused by 1,3-diaminopropane, produced during the metabolic degradation of spermidine (Eq. (2)). The inhibition constant for metabolized spermidine was determined using the plot v vs log [I]

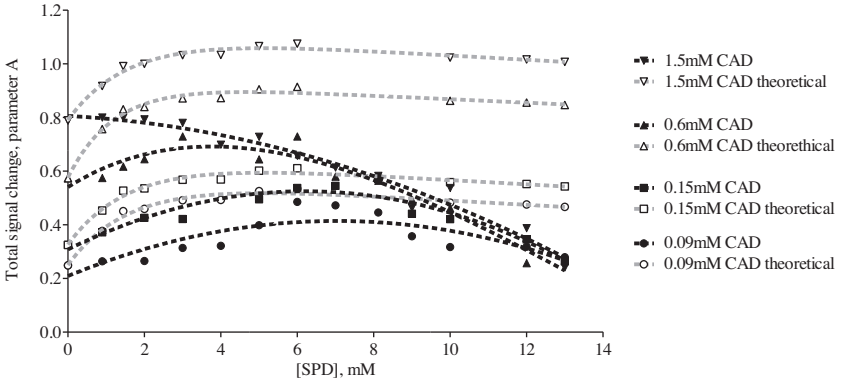

Fig. 2. The dependence of total signal change parameter *A* on spermidine concentration at different cadaverine concentrations (black dots and black lines). The experiments were carried out under constant stirring at 25.0 °C, pH 7.0 (0.1 M phosphate buffer), [PSAO] = 0.043 U/ml. The theoretical summarized effect of SPD and CAD if no additional inhibition by spermidine on cadaverine oxidation occurs is shown with gray dots and gray lines. Experimental points are the mean of at least 3 measurements.

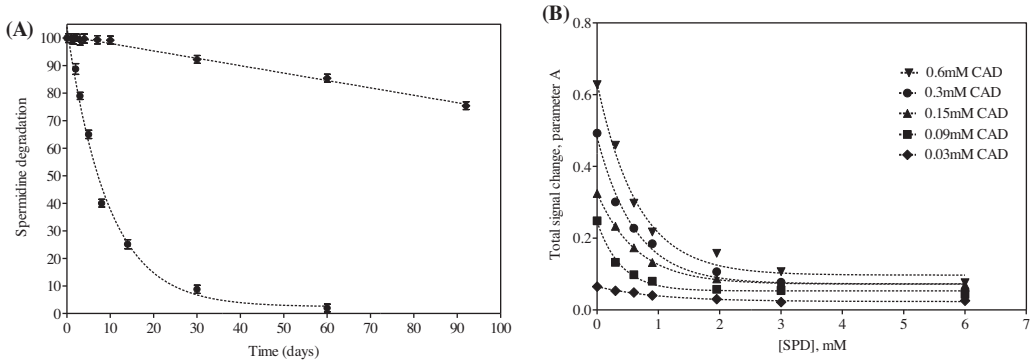

Fig. 3. (A) The degradation of the 3.0 mM spermidine solution: ♦ at 4 °C and ● at room temperature. (B) The dependence of the signal change parameter *A* of the one-month-old SPD solution at different CAD concentrations. The experiments were carried out under constant stirring at 25.0 °C, pH 7.0 (0.1 M phosphate buffer), [PSAO] = 0.043 U/ml. Experimental points are the mean of at least 3 measurements.

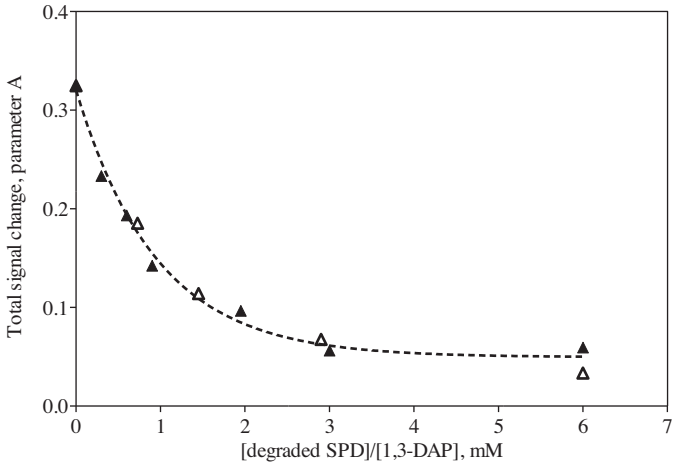

Fig. 4. The inhibiting effect of the degraded SPD (▲) and 1,3-DAP (Δ) on PSAO activity. The experiments were carried out under constant stirring at 25.0 °C, pH 7.0 (0.1 M phosphate buffer), [PSAO] = 0.043 U/ml, [CAD] = 0.15 mM. Experimental points are the mean of at least 3 measurements.

(data not shown) with Cheng–Prussoff equation. The value of K_i^{mSPD} for metabolized spermidine was 0.28 ± 0.05 mM.

ESI–MS spectra were investigated in order to get additional information about the composition of the solution of degraded spermidine. For MS spectra, approximately 300-fold dilutions were made in MeOH/water solution (8:2), with the final concentration of spermidine or its metabolites approximately 200 μg/g. Analysis of the MS spectrum indicated the same *m*/*z* values in 30-days old spermidine solution, which are characteristic to spermidine ESI–MS spectrum [48]: the main *m*/*z* 146 belonging to the protonated spermidine and other four *m*/*z* values (58, 72, 84, 112 and 129) resulting from spermidine fragmentation. In the spectrum of the degraded spermidine, *m*/*z* 75 and *m*/*z* 84 were additionally found, indicating the presence of 1,3-diaminopropane and Δ^1-pyrroline. The obtained spectrum is shown on Fig. S1.

We compared the inhibiting effect of the degraded spermidine and 1,3-diaminopropane on PSAO activity toward cadaverine on different cadaverine concentrations. The comparison of the inhibiting effects, shown as an example at 0.15 mM cadaverine, is on Fig. 4.

The effects of degraded spermidine and 1,3-diaminopropane are similar, indicating that both amines act as competitive inhibitors toward PSAO. For the calculation of the inhibition constant of 1,3-diaminopropane ($K_i^{1,3\text{-DAP}}$), we used the double reciprocal plot. The obtained value of $K_i^{1,3\text{-DAP}}$ was 0.43 ± 0.12 mM. This value is quite close to 0.11 mM, characterizing the 1,3-diaminopropane inhibiting effect on the oxidation of putrescine by diamine oxidase of human origin reported by Holinka and Gurpide [49]. The inhibiting effect of 1,3-diaminopropane on diamine oxidase from pea seedlings has been so far characterized only qualitatively [33–36].

4. Conclusion

The activity of diamine oxidase from pea seedlings is considerably influenced by spermidine, acting as an inhibiting substrate, and the spermidine metabolite 1,3-diaminopropane, acting as a competitive inhibitor. The inhibition of PSAO, increasing in time due to the decay of spermidine, decreases in turn the sensitivity of PSAO-based biosensors and can cause false-negative results of the detection of biogenic amines in their mixtures. So, if there is a considerable amount of arginine or ornithine present in proteins, which are undergoing putrefaction, PSAO-based biosensors are suitable for the analysis of biogenic amines only during the first week of protein putrefaction. In addition, time required to acquire results with biosensors, working in steady state mode, increases, as the initial rate of the reaction decreases in the presence of competitive inhibitors and there is a constant need for recalibration of the biosensor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2015.09.007.

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