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ACTIVITY AND KINETIC PROPERTIES OF ADENOSINE 5′-PHOSPHOSULFATE REDUCTASE IN THE INTESTINAL SULFATE-REDUCING BACTERIA

Adenosine 5'-phosphosulfate (APS) reductase activity and the kinetic properties of the enzyme from intestinal sulfate-reducing bacteria Desulfovibrio piger Vib-7 and Desulfomicrobium sp. Rod-9 has never been well-characterized and has not been studied yet. ***The aim*** *and background of this work was to investigate the* ***dissimilatory*** *APS reductase activity in cell-free extract of intestinal sulfate-reducing bacteria D. piger Vib-7 and Desulfomicrobium sp. Rod-9 and to carry out the kinetic analysis of enzymatic reaction.* ***Methods.*** *Microbiological, biochemical, and biophysical methods of the studies, and statistical processing of the results were used; the obtained data were compared with those from literature.* ***Results.*** *Dissimilatory APS reductase activity in the sulfate-reducing bacteria isolated from human intestine was studied. The highest activity of the enzyme (0.34±0.029 U×mg^{-1} protein) was measured in the cell-free extract prepared from D. piger Vib-7 cells then from Desulfomicrobium sp. Rod-9 (0.22±0.018 U×mg^{-1} protein). The optimal temperature (+35 ºC) and pH (8.0) for APS reductase reaction were determined. The analysis of the kinetic properties of the bacterial APS reductase was carried out. The APS reductase activity, initial (instantaneous) reaction rate (V_0) and maximum rate of the APS reductase reaction (V_{max}) in both D. piger Vib-7 and Desulfomicrobium sp. Rod-9 bacterial strains were defined. Michaelis constants (K_m) of the enzyme reaction (4.33±0.47 and 3.57±0.32 mM for D. piger Vib-7 and Desulfomicrobium sp. Rod-9, respectively) were determined.* ***Conclusion.*** *The described results of these studies can be the prospects to clarify the etiological role of these bacteria in the development of inflammatory bowel diseases humans and animals.*

K e y w o r d s : activity of APS reductase, kinetic analysis, sulfate-reducing bacteria, intestinal microbiocenosis, inflammatory bowel diseases, ulcerative colitis.

Sulfate-reducing bacteria produce hydrogen sulfide in the intestine lumen in the process of dissimilatory sulfate reduction. This process includes many stages where many different enzymes are involved [2, 7]. One of these enzymes is adenosine 5′-phosphosulfate (APS) reductase catalyzing the two-electron reduction of APS to sulfite and AMP, and it is a key step in the sulfate assimilation pathway in sulfate-reducing bacteria [13]. The adenylation product, APS, is reduced to sulfite, which is then further reduced to sulfide [2, 7, 13]. This final metabolic product is carcinogenic

to intestinal epithelial cells, and can cause inhibition of cytochrome oxidase, colonocytes oxidation of butyrate, destruction of epithelial cells, development of ulcers and inflammation with subsequent development of colon cancer [4, 8].

As far as it is aware, dissimilatory APS reductase from intestinal sulfate-reducing bacteria *D. piger* and *Desulfomicrobium* has never been well-characterized and has not been studied. In literature, there are some data about APS reductase of the sulfate-reducing bacteria isolated from environment [12, 13]. However, the data on activity of this enzyme from intestinal sulfate-reducing bacteria *Desulfovibrio piger* and *Desulfomicrobium* sp. has not been reported yet.

The aim of this work was to investigate the dissimilatory APS reductase activity in cell-free extract of intestinal sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 and to carry out the kinetic analysis of the enzymatic reaction.

Materials and Methods

The objects of the study were sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine and identified [9, 10].

Bacterial growth and cultivation. Bacteria were grown in the nutrition-modified Kravtsov-Sorokin's liquid medium [9]. Before bacteria seeding in the medium, 0.05 ml/l of sterile solution of $Na_2S \times 9H_2O$ (1%) was added. A sterile 10N solution of NaOH (0.9 ml/l) in the medium was used to provide the final pH 7.2. The medium was heated in boiling water for 30 min in order to obtain an oxygen-free medium, and then cooled to +30 °C. The bacteria were grown for 72 hours at +37 °C under anaerobic conditions. The tubes were brim-filled with medium and closed to provide anaerobic conditions.

Obtaining cell-free extracts. Cells were harvested at the beginning of the stationary phase, suspended at 4 °C in buffer containing 50 mM Tris·HCl (pH 8.0), and passed through a Manton-Gaulin press at 9000 psi. The extracts were centrifuged at 4 °C for 10 min at 15.000*g*; the pellet was then used as sedimentary fraction, and the supernatant obtained was termed the soluble fraction [5]. This extract was subjected to further centrifugation at 180.000*g* for 1 h to eliminate the membrane fraction. A clear supernatant, containing the soluble fraction, was then used as cell-free extract. Protein concentration in the cell-free extracts was determined by the Lowry method [11].

Assays for APS reductase activity. Enzyme assays were performed by using cell-free extracts, soluble and sedimentary fraction. The enzyme was assayed spectrophotometrically as described in paper [12]. Adenosine 5'-phosphosulfate reductase utilizes the AMP-dependent reduction of ferricyanide occurring in the presence of sulfite. The decrease in absorbance at 420 nm was followed at room temperature (+25 °C). The units of APS reductase were defined as μmoles of ferricyanide reduced per minute. Specific enzyme activity was expressed as $U \times mg^{-1}$ protein. The activity of the studied enzyme in the cell-free extracts of both bacterial strains under the effect of different temperature (+20, +25, +30, +35, +40, +45°C) and pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0) in the medium incubation was measured.

Kinetic analysis. Kinetic analysis of the enzyme reaction was performed in a standard incubation medium (as it was described above) with modified physical and chemical characteristics of the respective parameters (the incubation time, substrate concentration, temperature and pH). The kinetic parameters characterizing the ATP sulfurylase reaction are the initial (instantaneous) reaction rate (V_0), maximum rate of the reaction (V_{max}), maximum amount of the reaction product (P_{max}) and characteristic reaction time (time half saturation) τ were determined. The amount of the reaction product was calculated stoichiometrically. The kinetic parameters characterizing ATP sulfurylase reactions are Michaelis constant (K_m) and maximum reaction rate of substrate decomposition were determined by Lineweaver-Burk plot [6]. For analysis of the substrate kinetic mechanism of APS reductase, initial velocity rates were measured under standard assay conditions with substrate concentrations (APS). The resulting data were also analysed by global curve fitting in SigmaPlot (Systat Software, Inc.) to model the kinetic data to rapid equilibrium rate equations describing ordered sequential, $V=(V_{max}[A][B])/(K_A K_B+K_B[A]+[A][B])$, and random sequential, $V=(V_{max}[A][B])/(\alpha K_A K_B+K_B[A]+K_A[B]+[A][B])$, kinetic mechanisms, where V is the initial velocity, V_{max} is the maximum velocity, K_A and K_B are the K_m values for substrates A and B, respectively, and α is the interaction factor if the binding of one substrate changes the dissociation constant for the other [14].

Statistical analysis. Kinetic and statistical calculations of the results were carried out using the software MS Office and Origin computer programs. The research results were treated by the methods of variation statistics using Student *t*-test. The equation of the straight line that the best approximates the experimental data was calculated by the method of least squares. The absolute value of the correlation coefficient *r* was from 0.90 to 0.98. The statistical significance of the calculated parameters of line was tested by the Fisher's *F*-test. The accurate approximation was when $P \leq 0.05$ [1].

Results and Discussion

Activity of adenosine 5′-phosphosulfate reductase, an important enzyme in the process of dissimilatory sulfate reduction in sulfate-reducing bacteria, was measured in different fractions (cell-free extract, soluble, and sedimentary) obtained from *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 cells (Table 1). The results of this study showed that the highest specific activity of the enzyme was 0.51±0.053 and 0.17±0.016 U×mg^{-1} protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively, in soluble fraction. Slightly lower activity of APS reductase for both bacterial strains was detected in the cell-free extract compared to soluble fraction. Its values designated 0.34±0.029 U×mg^{-1} protein for *D. piger* Vib-7 and 0.11±0.012 U×mg^{-1} protein for *Desulfomicrobium* sp. Rod-9. There was not observed enzyme activity in sedimentary fraction.

The effect of temperature and pH of the incubation medium on the APS reductase activity in the cell-free extracts of the sulfate-reducing bacteria was studied (Fig. 1). The maximum specific activity for both bacterial strains was determined at +35 ºC.

Table 1

APS reductase activity in different fractions obtained from *Desulfovibrio piger* Vib-7 and *Desulfomicrobium sp.* Rod-9 cel

Sulfate-reducing bacteria	Specific activity of APS reductase (U×mg^{-1} protein)		
	Cell-free extract	Individual fractions	
		Soluble	Sedimentary
Desulfovibrio piger Vib-7	0.34±0.029	0.51±0.053	0
Desulfomicrobium sp. *Rod-9*	0.11±0.012**	0.17±0.016***	0

Comment: The assays were carried out at protein concentration of 48.12 mg/ml (for *D. piger* Vib-7) and 43.75 mg/ml (for *Desulfomicrobium* sp. Rod-9). Enzyme activity was determined after 30 min of incubation. Statistical significance of the values M±m, n = 3; **P<0.01, ***P<0.001, compared to *D. piger* Vib-7 strain.

An increase or decrease in temperature of incubation leads to a decrease of the activity of studied enzyme in the cell-free bacterial extracts. The highest enzyme activity of APS reductase was determined in the cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 at pH 8.0.

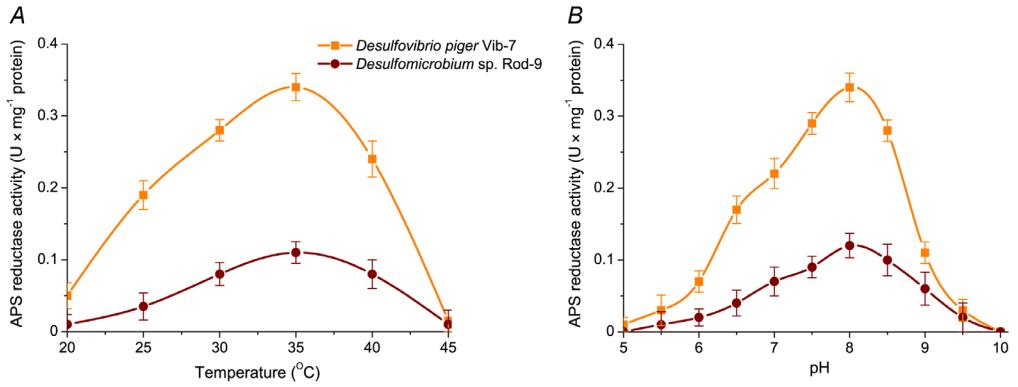

Fig. 1. Effect of temperature (A) and pH (B) on the APS reductase activity in the cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9

Thus, temperature and pH optimum of this enzyme with APS as a substrate was +35ºC and pH 8.0, respectively. The enzyme activity exhibited typical bell-shaped curves as a function of temperature and pH.

To study the characteristics and mechanism of APS reductase reaction, the initial (instantaneous) reaction rate (V_0), maximum rate of the reaction (V_{max}), maximum amount of reaction product (P_{max}) and reaction time (τ) were defined. Dynamics of ferricyanide reduction in the presence of sulfite in the cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 was studied for investigation of the kinetic parameters of APS reductase (Fig. 2).

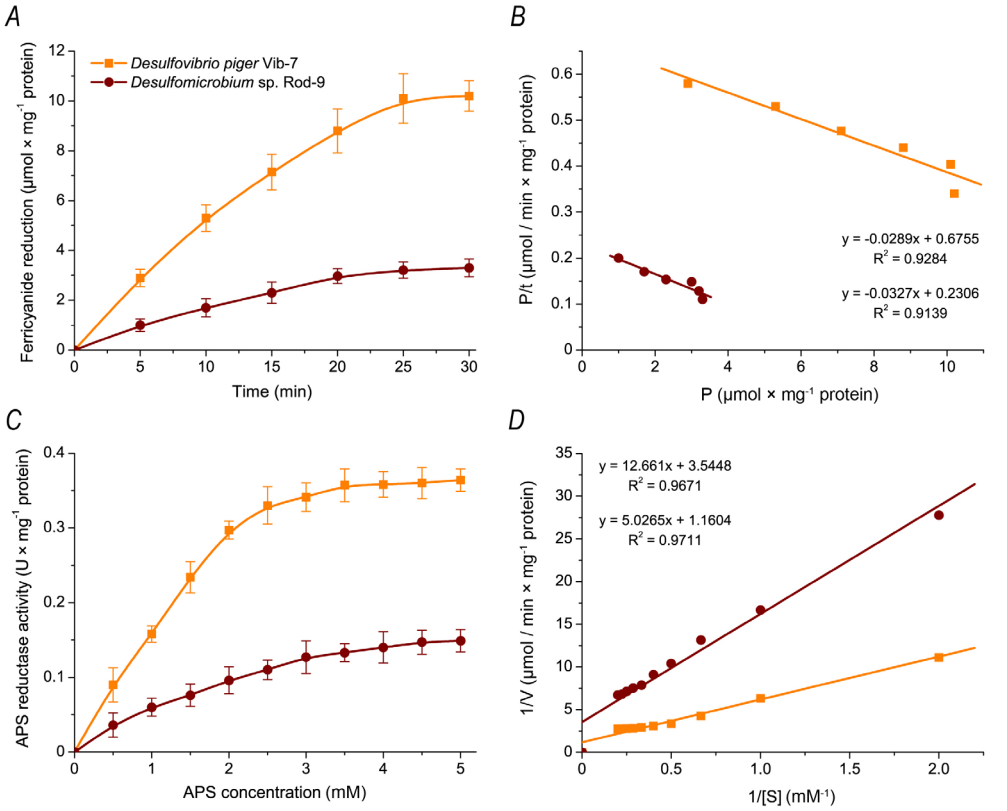

Fig. 2. Kinetic parameters of APS reductase activity in cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium sp.* Rod-9

A – dynamics of product accumulation (M ± m, n = 3); *B* – linearization of the curves of product accumulation in {P/t; P} coordinates (n = 3; $R^2 > 0.9$; F <0.02); *C* – the effect of different APS concentrations on APS reductase activity (M ± m, n = 3); *D* – linearization of concentration curves, which shown in Fig. 2*C*, in the Lineweaver-Burk plot, where V is rate of APS reductase reaction and S is substrate concentration (n = 3; $R^2 > 0.95$; F < 0.005).

Experimental data showed that the kinetic curves of APS reductase activity have tendency to saturation (Fig. 2*A*). The analysis of the results allows to reach the conclusion that the kinetics of APS reductase activity in cell-free extracts of the sulfate-reducing bacteria was consistent to the zero-order reaction in the range of 0–10 min (the graph of the dependence of product formation on the incubation time was almost linear in this interval of time). Therefore the duration of the incubation of bacterial cells extracts was 25 min in subsequent experiments.

Amount of product of APS reductase reaction in the *D. piger* Vib-7 was higher compared to the *Desulfomicrobium* sp. Rod-9 in the entire range of time factor. The basic kinetic properties of the reaction in the cell-free extracts of the sulfate-reducing bacteria were calculated by linearization of the data in the {P/t; P} coordinates (Fig. 2*B*, Table 2).

Table 2

Kinetic parameters of the product accumulation in the cell-free extracts of *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9

Kinetic parameters	Sulfate-reducing bacteria	
	***Desulfovibrio piger* Vib-7**	***Desulfomicrobium* sp. Rod-9**
V_0 (μmol/min×mg^{-1} protein)	0.675 ± 0.062	0.231 ± 0.022***
P_{max} (μmol×mg^{-1} protein)	23.37 ± 2.37	7.05 ± 0.71***
τ (min)	34.60 ± 3.42	30.58 ± 3.14

Comment: V_0 is initial (instantaneous) reaction rate; P_{max} is maximum amount (plateau) of the product of reaction; τ is the reaction time (half saturation period). Statistical significance of the values M ± m, n = 3; ***P<0.001, compared to the *Desulfovibrio piger* Vib-7 strain.

The kinetic parameters of APS reductase in cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were significantly different. The values of initial (instantaneous) reaction rate (V_0) for APS reductase activity in the cell-free extracts of both bacterial strains were calculated by the maximum amount of the product reaction (P_{max}). As shown in Table 2, V_0 for APS reductase reaction was higher in the cell-free extracts of *D. piger* Vib-7 (0.675 ± 0.062 μmol/min×mg^{-1} protein) compared to *Desulfomicrobium* sp. Rod-9 (0.231 ± 0.022 μmol/min×mg^{-1} protein). Based on these data, there is an assumption that the *D. piger* Vib-7 can consume APS much faster (approximately three times) in their cells than a *Desulfomicrobium* sp. Rod-9. Moreover, this hypothetical assumption can be also confirmed by obtained data on the product accumulation in enzymatic reaction, its maximum values designated 23.37±2.37 μmol×mg^{-1} protein for *D. piger* Vib-7 and 7.05±0.71 μmol×mg^{-1} protein for *Desulfomicrobium* sp. Rod-9. However, the reaction time (half saturation period) for APS reduction was almost similar in both bacterial strains.

The kinetic analysis of APS reductase activity dependence on the substrate concentration was executed. According to the obtained results, increasing of sulfite concentrations from 0.5 to 5.0 mM causes a monotonic rise of the studied enzyme activity and the activity was maintained on unchanged level (plateau) under substrate concentrations over 5.0 mM (Fig. 2*C*). The curves of the dependence {1/V; 1/[S]} were distinguished by the tangent slope and intersect the vertical axis in one point (Fig. 2*D*). The basic kinetic parameters of APS reductase activity in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 cell-free extracts were identified by linearization of the data in the Lineweaver-Burk plot (Table 3).

Calculation of the kinetic parameters of APS reductase activity indicates that the maximum rate (V_{max}) of product accumulation in the cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 was significantly different from each other. The maximum rate of APS reaction for *D. piger* Vib-7 was designated 0.862±0.084

μmol/min×mg^{-1} protein and 0.282±0.027 μmol/min×mg^{-1} protein for *Desulfomicrobium* sp. Rod-9 strain. In this case, Michaelis constants (K_m) of APS reductase for both bacterial strains were also different: 4.33 ± 0.47 and 3.57 ± 0.32 mM for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively (Table 3).

Table 3

Kinetic parameters of APS reductase activity dependence on APS concentration in the incubation medium

Kinetic parameters	Sulfate-reducing bacteria	
	***Desulfovibrio piger* Vib-7**	***Desulfomicrobium* sp. Rod-9**
V_{max} (μmol/min×mg^{-1} protein)	0.862 ± 0.084	0.282 ± 0.027***
K_m (mM)	4.33 ± 0.47	3.57 ± 0.32

Comment: V_{max} is maximum rate of the enzyme reaction; K_m is Michaelis constant, determined by substrate. Statistical significance of the values M ± m, n = 3; ***P<0.001, compared to the *Desulfovibrio piger* Vib-7 strain.

The obtained parameters of APS reductase reaction in the cell-free extracts of *D. piger* Vib-7 were differed to data on the enzymatic activity from *P. aeruginosa* defined previously by Bick et al. for APS reductase [3]. The authors have shown that pH and temperature optimum for studied enzyme is 8.5 and +30 ºC, respectively. However, in our case, pH and temperature optimum for APS reductase activity was 8.0 and +35 ºC. These data correspond to conditions which are present in the human large intestine from where the bacterial strains were isolated. Perhaps, such conditions provide their intensive development in the gut. Initial enzyme reaction velocity of *P. aeruginosa* was also carried out by Bick et al. to determine the kinetic constants and to compare its activity with that of *E. coli.* The V_{max} was enzyme reaction was 5.8 μmol×min^{-1}×mg^{-1} protein and the K_m[APS] was 1.75 μM [3].

In summary, the APS reductase activity, V_0, maximum amount of the product of reaction, APS reaction time (half saturation period), V_{max} and Michaelis constants were significantly higher in the *D. piger* Vib-7 cells compared to the *Desulfomicrobium* sp. Rod-9 strain. The maximum APS reductase activity for both strains has been determined at +35 ºC and at pH 8.0. The increase or decrease in temperature and pH of incubation leads to decrease of the activity of studied enzyme in the cell-free bacterial extracts. The kinetic parameters of APS reductase reaction depended on the substrate concentration. According to the obtained results, increasing of APS concentrations from 0.5 to 5.0 mM causes a monotonic rise of studied enzyme activity and the activity was maintained on an unchanged level (plateau) under substrate concentrations over 5.0 mM. Michaelis constants (K_m) of the enzyme reaction (4.33±0.47 and 3.57±0.32 mM for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively) were determined. The studies of the physiological and biochemical

properties of the intestinal sulfate-reducing bacteria, the process of the dissimilatory sulfate reduction, in particular participation of APS reductase in this process, the activity and kinetic properties of this enzyme in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 bacterial strains, their production of hydrogen sulfide in detail can be perspective for clarification of the etiological role of these bacteria in the development of various diseases of the gastrointestinal tract in humans and animal.

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АКТИВНІСТЬ ТА КІНЕТИЧНІ ВЛАСТИВОСТІ АДЕНОЗИН-5'-ФОСФОСУЛЬФАТРЕДУКТАЗИ КИШКОВИХ СУЛЬФАТВІДНОВЛЮВАЛЬНИХ БАКТЕРІЙ

Реферат

Аденозин-5'-фосфосульфат (АПС) редуктаза та кінетичні властивості цього ферменту кишкових сульфатвідновлювальних бактерій Desulfovibrio piger Vib-7 і Desulfomicrobium sp. Rod-9 ніколи не були добре охарактеризовані й досі недосліджені. **Мета роботи** *дослідити активність дисиміляційної АПС-редуктази у безклітинних екстрактах кишкових відновлювальних бактерій D. piger Vib-7 і Desulfomicrobium sp. Rod-9, а також виконати кінетичний аналіз реакції досліджуваного ферменту.* **Методи**. *У цій роботі використані мікробіологічні, біохімічні та біофізичні методи досліджень, а також застосована статистична обробка результатів; отримані результати порівняні з даними літератури.* **Результати**. *Досліджено активність дисиміляційної АПС-редуктази сульфатвідновлювальних бактерій, виділених з кишечника людини. Найвища активність цього ферменту (0,34±0,029 U×мг$^{-1}$ білка) виміряна у безклітинному екстракті, отриманого з клітин D. piger Vib-7, а ніж зі штаму Desulfomicrobium sp. Rod-9 (0,22±0,018 U×мг$^{-1}$ білка). Визначено оптимальну температуру (+35°С) і pH (8,0) для реакції досліджуваного ферменту. Проведено аналіз кінетичних властивостей бактеріальної АПС-редуктази. Досліджено активність АПС-редуктази, початкову (миттєву) швидкість реакції (V_0) і максимальну швидкість АПС-редуктазної реакції (V_{max}) в обох бактеріальних штамів D. piger Vib-7 і Desulfomicrobium sp. Rod-9. Визначено константи Міхаеліса (K_m) для ферментативної реакції (4.33±0,47 і 3,57±0,32 мМ для D. piger Vib-7 і Desulfomicrobium sp. Rod-9, відповідно).* **Висновок**. *Результати досліджень можуть бути перспективними для з'ясування етіологічної ролі цих бактерій у розвитку запальних захворювань кишечника людини і тварин.*

Ключові слова: активність АПС-редуктази, кінетичний аналіз, сульфатвідновлювальні бактерії, кишкові мікробіоценози, запальні захворювання кишечника, виразковий коліт.

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АКТИВНОСТЬ И КИНЕТИЧЕСКИЕ СВОЙСТВА АДЕНОЗИН-5'-ФОСФОСУЛЬФАТРЕДУКТАЗЫ КИШЕЧНЫХ СУЛЬФАТВОССТАНАВЛИВАЮЩИХ БАКТЕРИЙ

Реферат

Аденозин-5'-фосфосульфат (АПС) редуктаза и кинетические свойства этого фермента кишечных сульфатвосстанавливающих бактерий Desulfovibrio piger Vib-7 и Desulfomicrobium sp. Rod-9 никогда не были хорошо охарактеризованы и до сих пор неисследованные. **Целью работы** *было изучение активности диссимиляционной АПС-редуктазы в бесклеточных экстрактах кишечных сульфатвосстанавливающих бактерий D. piger Vib-7 и Desulfomicrobium sp. Rod-9, а также выполнить кинетический анализ реакции исследуемого фермента.* **Методы**. *В работе использованы микробиологические, биохимические и биофизические методы исследований, а также применена статистическая обработка результатов; полученные результаты сравнены с данными литературы.* **Результаты**. *Исследовано диссимиляционую АПС-редуктазу сульфатвосстанавливающих бактерий, выделенных из кишечника человека. Самая высокая активность этого фермента (0,34±0,029 U×мг$^{-1}$ белка) измерена в бесклеточном экстракте, полученного из клеток D. piger Vib-7, чем из штамма Desulfomicrobium sp. Rod-9 (0,22±0,018 U×мг$^{-1}$ белка). Определено оптимальную температуру (+35 ºС) и pH (8,0) для реакции исследуемого фермента. Проведено анализ кинетических свойств бактериальной АПС-редуктазы. Исследовано активность АПС-редуктазы, начальную (мгновенную) скорость реакции (V_0) и максимальную скорость АПС-редуктазной реакции (V_{max}) в обоих бактериальных штаммов D. piger Vib-7 и Desulfomicrobium sp. Rod-9. Определено константы Михаэлиса (K_m) для ферментативной реакции (4.33±0,47 и 3,57±0,32 мМ для D. piger Vib-7 и Desulfomicrobium sp. Rod-9, соответственно).* **Вывод.** *Результаты исследований могут быть перспективными для выяснения этиологической роли этих бактерий в развитии воспалительных заболеваний кишечника человека и животных.*

Ключевые слова: активность АПС-редуктазы, кинетический анализ, сульфатвосстанавливающие бактерии, кишечные микробиоценозы, воспалительные заболевания кишечника, язвенный колит.

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