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RECOMBINANT FORMS OF ARGINASE AND ARGININE DEIMINASE AS CATALYTIC COMPONENTS OF *ARGITEST* ENZYMATIC KIT FOR L-ARGININE ANALYSIS

For the time, no screening test kits for L-arginine monitoring in biological fluids, including the blood, have been available in the arsenal of domestic clinical diagnostics. The Argitest enzymatic kit is based on the enzymatic-chemical method of L-arginine quantitative analysis developed using different forms of recombinant human liver arginase I (arginase) and arginine deiminase. The method is highly selective, cheap, simple, and fast. It is remarkable for stability of enzymes and final products. The article demonstrates the possibility of using recombinant arginine-selective enzymes in composition of Argitest kit. The catalytic and analytic characteristics of these enzymes have been studied. It has been shown that each enzyme can be used as a component of enzymatic kit.

Keywords: L-arginine, arginase I, arginine deiminase, 2,3-butanedione monoxime, urease, and Argitest enzymatic kit.

L-arginine amino acid (hereinafter, Arg), a precursor of L-ornithine, L-citrulline, L-glutathione, γ-aminobutyric acid, spermidine, and other compounds, is one of the most polarized positively charged amino acids [1]. Arg metabolism can run in, at least, two alternative ways: 1) oxidative (involving NO-synthase) with the formation of L-citrulline and NO; and 2) non-oxidative (with arginase I involved) with the formation of L-ornithine and urea. The simultaneous occurrence of both processes is also possible [2]. Almost the entire chain of Arg transformations into derivative compounds occurs in the three organs: intestines, liver, and kidneys [3].

Arg content is usually determined by methods of ion exchange chromatography, fluorometry, spectrophotometry, capillary electrophoresis, polarography, flow-injection analysis, laser fluorescence spectroscopy and others [4—5]. The most widely used methods for Arg quantitative analysis are chromatographic ones, including high-performance liquid chromatography (HPLC). HPLC is based on sample pretreatment with derivatizing reagents followed by separation of these derivatives using chromatographic methods and determination of these compounds by tandem mass spectrometry (TMS) and fluorescence detection (FL). Although the HPLC method is sufficiently sensitive, reproducible and enables to define the entire set of amino acids (15—20 μl plasma), it has some drawbacks that limit its widespread use, especially, for rapid diagnosis. First of all, the HPLC requires expensive equipment and reagents, which makes analysis expensive for routine clinical purpose. The method requires a significant amount of high quality organic solvents and expensive derivatizing reagents. Secondly, the sample preparation and

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analysis take a long time and require 1— 2 days from the moment of derivatization to obtain quantitative data [5].

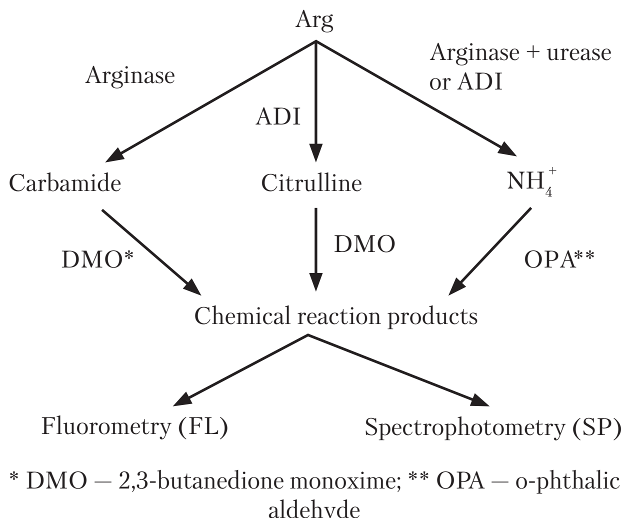
In analytical biotechnology, there are limited data on enzymatic methods for detecting Arg. The most promising enzymes for the development of such methods are Arg metabolic enzymes, in particular, arginase I, arginine deiminase (ADI), and arginine decarboxylase. Existing commercial enzymatic test systems (manufactured by *Sigma*, *Enzytec*™, *Elisa*, *Megazyme* and others) are mainly the multi-enzyme ones with spectrophotometric (SP) detection of final reaction product [6—8]. The multi-enzyme Arg detection methods have several drawbacks, including non-absolute selectivity to the target analyte caused by a positive reaction to guanidine compounds. The necessity to use a cascade of several (3—5) enzymes and exogenous co-factors increases the cost of methods and complicates the analysis procedure.

In previous studies, the authors have proposed biosensor approaches [9] and cost effective enzymatic and chemical methods for Arg detection [9—16] using one or two enzymes. Fig. 1 shows a schematic diagram of developed methods and Table 1 gives their analytical characteristics as compared with similar parameters of known multi-enzyme methods.

The methods indicated in Fig. 1 are based on the use of recombinant arginine hydrolyzing enzymes — human liver arginase I (arginase[1]) and bacterial arginine deiminase (ADI) obtained by own-developed methods from cells of recombinant microbial producers created by the authors (Table 2).

As Table 1 shows, the arginase-DMO method has several advantages over the other methods, including a high sensitivity and a wide range of Arg concentrations tested. This method does not require any prior Arg separation from samples. It is simple, highly selective, reliable, and commercially available; its practicability has been demonstrated on samples of industrial pharmaceuticals and commercial wines [9, 11].

For the time being, no test systems for monitoring Arg in biological fluids, including the blood,



* DMO — 2,3-butanedione monoxime; ** OPA — o-phthalic aldehyde

Fig.1. Enzymatic-chemical Arg analysis methods

have been available in the toolkit of national clinical diagnostics. In 2015, the authors were involved in implementing project research for R&D program of the NAS of Ukraine «Development, Testing, and Production of Pilot Series of *Argitest* Enzymatic Test Kit for Analyzing Arginine in Clinical Samples. Section 1. Optimization of Kit Composition, Analysis Conditions and Elaboration of Specifications». As a result of this project, the arginase-DMO method has been successfully tested on blood samples. Based on arginase-DMO enzymatic-chemical method *Argitest* enzymatic kit has been created for Arg identification and the specifications for it have been elaborated.

One of the objectives of the above research project was to determine the optimal enzyme component of *Argitest* kit. The objects studied were recombinant arginine hydrolyzing enzymes (Table 2) isolated and described by the authors.

MATERIALS AND METHODS OF THE RESEARCH

Recombinant microorganism strains from the museum of the Institute of Cell Biology of the NAS of Ukraine were used for this research. Recombinant yeast and bacteria cells have been created in previous studies and used as sources of target recombinant enzymes (Table 2).

Table 1

Comparison of Enzymatic Chemical Methods for Arg Detection

Method	Enzyme	Linear range, μM	Sensitivity, μM	Source
Known enzymatic methods				
Multi-enzymatic, SP, λ = 340 nm	Arginase, urease, glutamate dehydrogenase	Up to 470	2.0	[6]
Multi-enzymatic, SP, λ = 340 nm	Arginase, urease, glutamate dehydrogenase	2.9—100	2.1	[7]
Multi-enzymatic, SP, λ = 555 nm	ADI, arginine succinate synthase; pyruvate phosphate dikinase; pyruvate oxidase, horseradish peroxidase	Up to 100	—	[8]
Methods developed by authors of this research paper				
ДМО-SP, λ = 480 nm	Arginase	7—100	5.0	[9—11]
ДМО-FL, λ_{ex} = 360 nm, λ_{em} = 510 nm	Arginase	0.06—200	0.04	[9, 11, 12]
OPA-SP, λ = 362 nm	Arginase -urease	0.9—60.0	0.85	[13]
OPA-FL, λ_{ex} = 364 nm, λ_{em} = 425 nm	Arginase-urease	0.09—6.0	0.08	[14]
OPA-FL, λ_{ex} = 364 nm, λ_{em} = 415 nm	ADI	0.35—24	0.25	[15]
OPA-SP, λ = 340 nm	ADI	0.7—50	0.55	[16]

DMO — 2,3-butanedione monoxime; SP — spectrophotometric detection of final product; FL — fluorometric detection of final product; OPA — o-phthalic aldehyde

Specific activity of recombinant enzyme (hereinafter — the enzymes) is expressed in μmol of product formed in 1 min per 1 mg protein ($\mu mol \cdot min^{-1} \cdot mg^{-1}$) under typical reaction conditions. Arginase activity was determined by the rate of urea formation [17—19]. ADI activity was analyzed in two stages determining the rate of ammonia formation [20, 21].

The intracellular enzymes arginase[1] and arginase[2] were isolated from cell-free extracts (CE), the secreted enzyme (arginase[3]) was extracted from culture fluid of corresponding recombinant microbial producers and furtherly purified by column chromatography by developed schemes (see references in Table 2).

Synthesized affine arginine-macro-porous glass sorbent was used to isolate arginase[1]. The enzyme preparations with a specific activity of 1500—2600 units per 1 mg protein were obtained by 40—60-fold purification with 42% yield for the most successful fractions. The preparations were stored at — 10 °C in 50 mM Tris-HCl buffer (TB), *pH* = 8.0, containing 1M NaCl, 1 mM $MnCl_2$, and 10% glyceryl. They kept their activity within the year and after 3 years of storage their enzymatic activity decreased twice only.

Affine chromatography on Ni-NTA-Superflow (Qiagen) sorbent was used to isolate arginase[2] and arginase[3]. Yield of enzymes with the highest activity accounted for 70% (6 mg/l culture fluid) and 35% (5 mg/l culture), respectively. Arginase[2] and arginase[3] preparations were stored at —20 °C keeping their activity fixed within the year in Tris-phosphate buffer, *pH* = 8.0 and phosphate buffer, *pH* = 7.2, respectively. Both buffers contained 0.15 M NaCl and 20% glycerol.

Table 2

Arginine Hydrolyzing Enzymes

Enzyme name	Symbol	Enzyme properties	Enzyme source	Source
Human liver original arginase I	Arginase[1]	Mn^{2+}-dependent, intracellular	*NCYC 495 Hansenula polymorpha pGAP1-HsARG1 leu2car1:ScLEU2*	17
Human liver $(His)_6$-tagged arginase I	Arginase[2]	Mn^{2+}-dependent, intracellular	*Saccharomyces cerevisiae 303/pYEX-4-ARG1 (MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100)*	18
Modified human liver arginase I	Arginase[3]	Co^{2+}-dependent, secreted	*H. polymorpha pFDM-ScMFa-HIS6-HsARG1*	19
Arginine deiminase *Mycoplasma hominis*	ADI	Intracellular	*Escherichia coli BL-21(pET3d-ADI)*	20, 21

Arginine deiminase (ADI) was extracted from insoluble «inclusion bodies» and purified by anion and hydrophobic chromatography on QAE-Sepharose and phenyl-Sepharose adsorbents, respectively. The resulting homogeneous ADI preparation obtained by electrophoresis was stored at 4 °C in 20 mM phosphate buffer, *pH* = 8.0, containing 1 M NaCl, without loss of activity within 20 months.

The catalytic and analytical properties of highly refined enzymes have been studied. The optimal conditions of enzymatic and chemical reactions and procedures for recording the final products have been described in detail in previous publications [9—11]. As a result, calibration curves of analytical signal (radiation intensity or light absorption of reaction mixture), i.e. response to substrate enzymatic conversion, dependence on Arg concentration have been built. To build the calibration curves, 0.1 ml Arg standard solutions in 30 mM, TB *pH* = 8.8, were placed glass tubes. The reaction was activated by adding 0.01 ml enzyme in 30 mM TB, *pH* = 8.8, up to a concentration of 1.5—3.0 unit/ml. The incubation mixture was stirred and kept for 10 minutes at 37 °C. Later, 3 ml 0.5% 2,3-DMO solution in 1.7 M H_2SO_4 was added to the mixture and heated in a water bath for 50 minutes. Optical density (by *SHIMADZU UV-1650 PC* spectrophotometer) or fluorescence intensity of the final reaction product (by *TECAN Infinite H200* fluorometer with excitation wave at 360 nm) was recorded as compared with the reference sample (0.1 ml 30 mM TB, *pH* = 8.8, instead of Arg solution).

The experiments were repeated from four to six times and the measurements were made in 3 parallels. For each sample, arithmetic mean (*M*), variance, standard deviation of average, relative standard deviation, and confidence interval were determined. The statistical parameters and diagrams were calculated and built using *Origin 8.0*. The curves were linearized by the regression equation $Y = A + BX$ (where *A* and *B* are equation parameters); correlation coefficient *R* and confidence interval of linear regression *p* were calculated. Parameter *B* corresponds to the slope ratio that is used for sensitivity determination. Coefficient *A* is a measure of background effect of additional ingredients on arginine identification, i.e. indicates the method selectivity [22]. The parameters and statistical data are given in Figures and Tables.

RESULTS AND DISCUSSION

Schematic diagram of reactions underlying the enzymatic-chemical methods used for Arg determination using arginase is shown in Fig. 2. The method is based on enzymatic hydrolysis of Arg to L-ornithine and urea (enzymatic reaction, step 1). Urea reacts with DMO at the chemical stage of reaction (step 2) with the formation of product that quantitatively measured by spectropho-

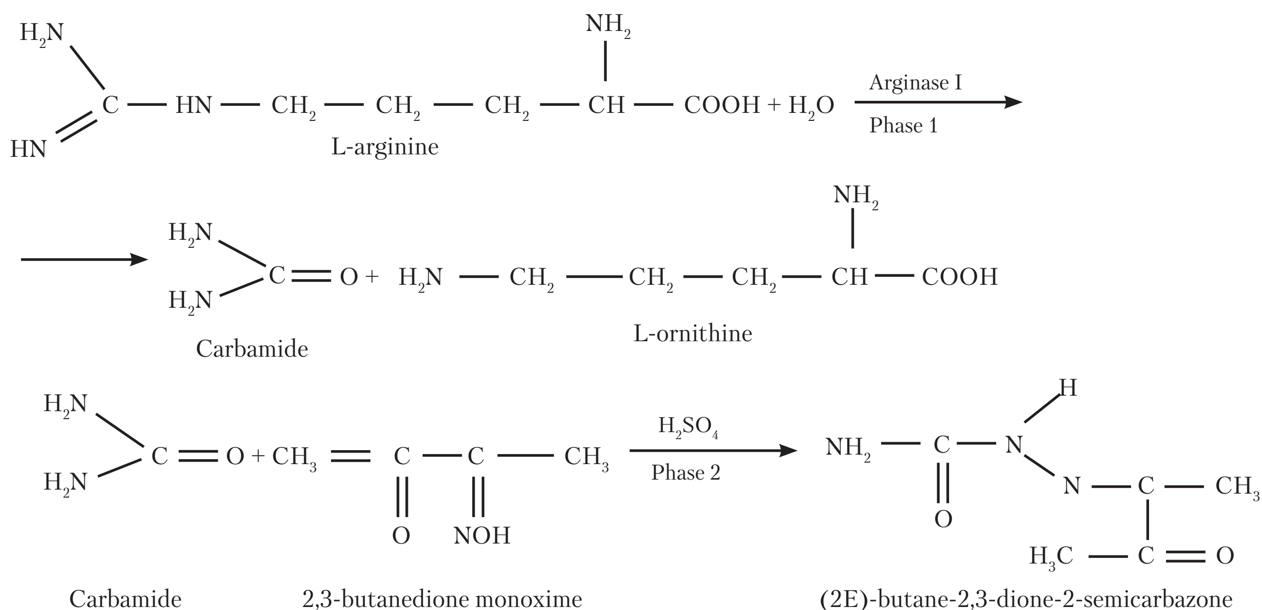


Fig. 2. General reaction scheme for Arg determination by carbamide formation using Arginase-DMO enzymatic-chemical method

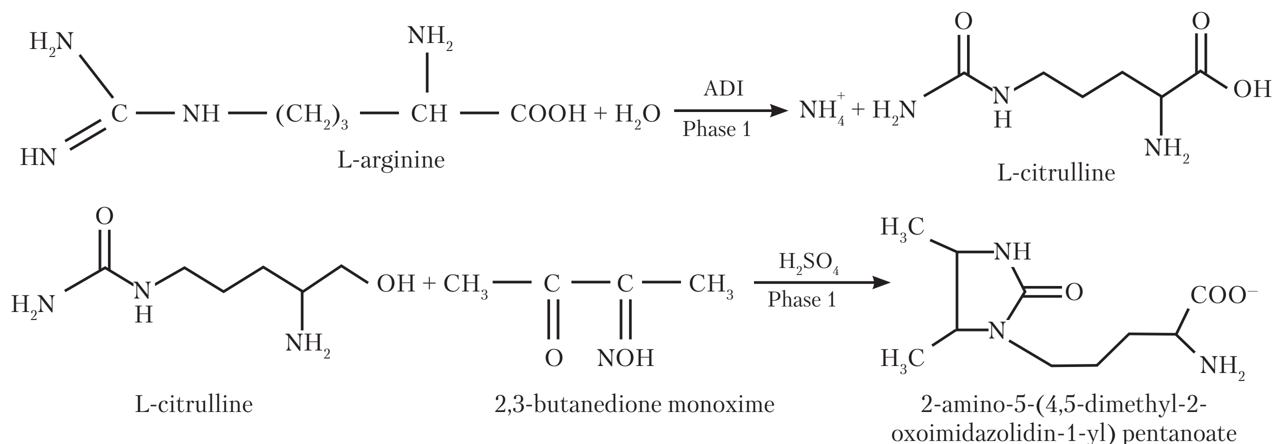


Fig. 3. General reaction scheme for Arg determination by citrulline formation using ADI-DMO enzymatic-chemical method

tometry (SP), at λ = 480 nm, or by fluorometry (FL), at 510 nm with excitation wave at 360 nm [11]. The final product of chemical reaction is (2E)-butane-2,3-dione-2-semicarbazone and other compounds, including cyclic ones.

ADI application to Arg determination by the enzymatic method provides highly selective and effective Arg degradation to citrulline. Schematic diagram of reactions underlying ADI-DMO method for Arg determination (for citrulline formation) is shown in Fig. 3. Concentration of final product of chemical reaction is measured by the spectrophotometry (SP), by optical density, at λ = 490 nm and by the fluorometry (FL),

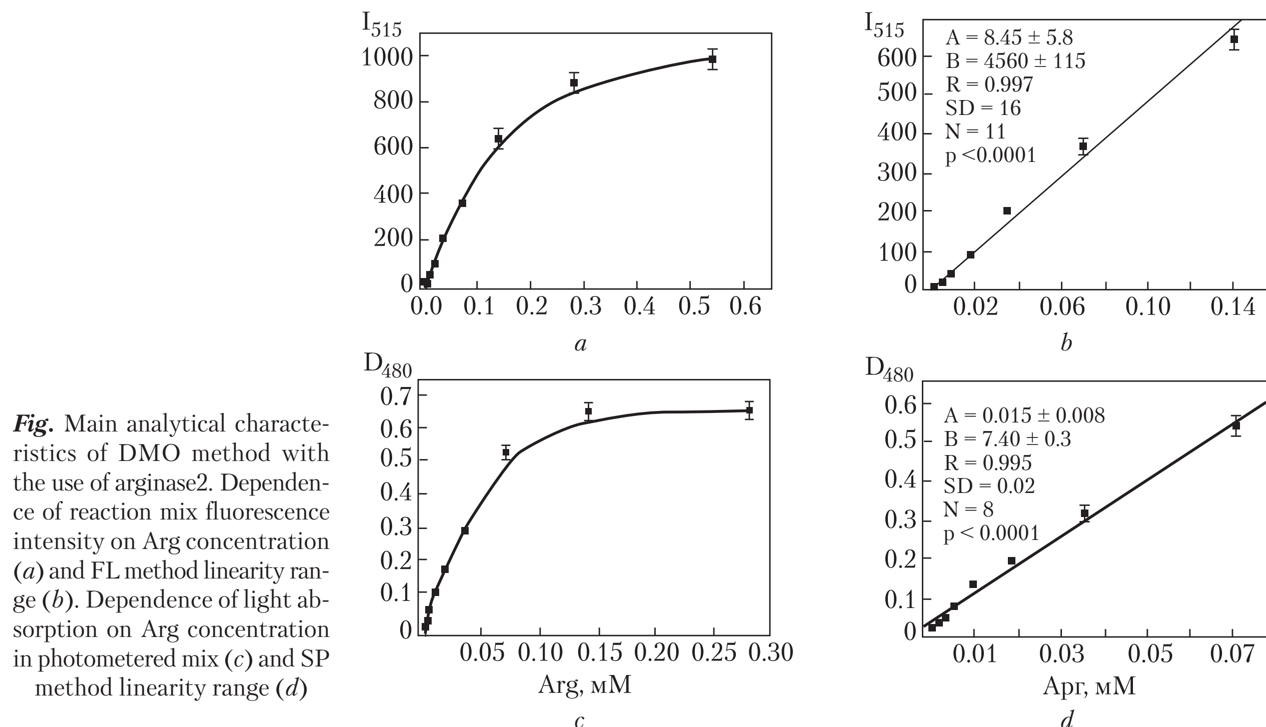


Fig. Main analytical characteristics of DMO method with the use of arginase2. Dependence of reaction mix fluorescence intensity on Arg concentration (*a*) and FL method linearity range (*b*). Dependence of light absorption on Arg concentration in photometered mix (*c*) and SP method linearity range (*d*)

at 515 nm, with excitation wave at 360 nm [15—16].

The optimal enzyme was selected by comparing the analytical characteristics of studied enzymes (Table 2) under optimized analysis conditions [11]. To this end, the dependence of fluorescence intensity and light absorption of reaction mixture on Arg concentration in the final sample was studied. Fig. 4 shows graphical results of studying the dependence of fluorescence intensity (*a, b*) and light absorption (*c, d*) of reaction mixture on Arg concentration using arginase[2]. Similar graphs were obtained from processing the experimental data using other enzymes.

Table 3 summarizes the analytical characteristics (calibration curve linearity and threshold sensitivity) of enzymatic and chemical DMO method for each enzyme studied.

The above data show that the properties of enzyme preparations, when used for the DMO method, are close, especially in FL record of reaction products. Arginase[2] was used as catalytic component in *Argitest* analytical kit. The *Argitest* kit with spectrophotometric and fluorometric detection of reaction products has been tested on human blood serum samples. The normal ranges and average values of Arg content for healthy people have been established. They are in good agreement with literature data [23].

Table 3

Key Analytical Parameters of DMO Method in the Case of Various Enzymes

Enzyme	Parameter			
	Linear range, μM		Sensitivity, μM	
	FL	SP	FL	SP
Arginase[1]	0.06—280	7—100	0.04	5.0
Arginase[2]	0.28—140	1.1—70	0.15	0.28
Arginase[3]	0.55—140	0.55—140	0.30	0.30
ADI	0.55—140	4.4—280	0.30	2.5

CONCLUSIONS

For the first time, the analytical characteristics of recombinant arginine-hydrolyzing enzymes as catalytic components of *Argitest* analytical kit have been studied. The principle of *Argitest* kit operation is enzymatic conversion of L-arginine and spectrophotometric or fluorometric detection of final product of chemical reaction of urea (under the action of arginase) or citrulline (under the action of ADI) with DMO. It has been shown that each studied enzyme can be used to determine the L-arginine. However, for creating *Argitest* kit, $(His)_6$-tagged human liver arginase I (arginase[2]) was as catalytic component. The commercial production of cost-effective *Argitest* analytical kit is expected to enable launching the monitoring of Arg content in human blood in the national clinical practice. In addition, the kit has good prospects for being used in veterinary medicine, food and pharmaceutical industries.

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РЕКОМБІНАНТНІ ФОРМИ АРГІНАЗИ
ТА АРГІНІНДЕІМІНАЗИ
ЯК КАТАЛІТИЧНІ СКЛАДОВІ
ЕНЗИМАТИЧНОГО НАБОРУ «АРГІТЕСТ»
ДЛЯ АНАЛІЗУ L-АРГІНІНУ

На сьогодні скринінгові тест-системи для моніторингу L-аргініну у біологічних рідинах, зокрема, у крові, в арсеналі засобів вітчизняної клінічної діагностики відсутні. Ензиматичний набір «Аргітест» базується на розробленому ензиматично-хімічному методі кількісного аналізу L-аргініну з використанням різних форм рекомбінантної аргінази I печінки людини та аргініндеімінази. Метод є високоселективним, економічно вигідним, простим та швидким у виконанні, відзначається стабільністю препаратів ензимів та продукту реакції. У статті доведено можливість використання препаратів рекомбінантних аргініно-селективних ензимів у складі набору «Аргітест». В результаті досліджень вивчено каталітичні та аналітичні характеристики цих препаратів та показано, що кожен з них може бути використано як складову ензиматичного набору.

Ключові слова: L-аргінін, аргіназа I, аргініндеіміназа, 2,3-бутандіонмонооксим, уреаза, ензиматичний набір «Аргітест».

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РЕКОМБИНАНТНЫЕ ФОРМЫ АРГИНАЗЫ
И АРГИНИНДЕИМИНАЗЫ
КАК КАТАЛИТИЧЕСКИЕ КОМПОНЕНТЫ
ЭНЗИМАТИЧЕСКОГО НАБОРА «АРГИТЕСТ»
ДЛЯ АНАЛИЗА L-АРГИНИНА

В настоящее время скрининговые тест-системы для мониторинга L-аргинина в биологических жидкостях, в том числе, в крови, в отечественной клинической диагностике отсутствуют. Энзиматический набор «Аргитест» базируется на разработанном энзиматическо-химическом методе количественного анализа L-аргинина с использованием разных форм рекомбинантной аргиназы I печени человека и аргининдеиминазы. Метод является высокоселективным, экономично выгодным, простым и быстрым в исполнении, отличается стабильностью энзиматических препаратов и продукта реакции. В статье продемонстрирована возможность использования препаратов рекомбинантных аргинин-селективных энзимов в составе набора «Аргитест». В результате исследований определены каталитические и аналитические характеристики энзиматических препаратов и показано, что каждый из них может быть использован как компонент энзиматического набора.

Ключевые слова: L-аргинин, аргиназа I, аргининдеиминаза, 2,3-бутандионмонооксим, уреаза, энзиматический набор «Аргитест».