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DEVELOPMENT OF SPECIFIC PRIMERS FOR 16S rRNA GENE ANALYSIS IN THE DETECTION OF *Ralstonia solanacearum* SPECIES COMPLEX

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Members of *Ralstonia solanacearum* species complex (RSSC) are causal agents of vascular wilt disease in more than 450 crop species, including solanaceous plants such as potatoes, tomatoes, bell pepper, eggplant, etc. These phytopathogens cause serious yield loss mostly in solanaceous crops which are grown in tropical, subtropical, and temperate regions of the world. Yield losses comprise 80–100% in potato, up to 91% for tomato, 10–30% in tobacco, 33–90% in banana, and reduce crop productivity and yield. PCR-methods are specific, sensitive and cost-effective approaches for the detection and identification of RSSC members.

The *objective* of this study was to compare specificity of routinely used primer mix for PCR RSSC detection with the newly developed pairs of species-specific primers for ease of use diagnostics in a laboratory.

Materials and Methods. The conserved genomic regions of the 16S rRNA sequences of *R. solanacearum*, *R. pseudosolanacearum*, and *R. syzygii* were selected for the design of primers for this study. Newly created primer species specificity was tested in PCR using the DNA of the two targets and 12 non-target strains of bacteria.

Results. Three pairs of newly created primers Rs-28(F)/Rs-193(R), Rs-28(F)/OLI-160(R), Rs28(F)/OLI248(R) produced single specific fragments for bacterial strains of *Ralstonia solanacearum*: 166 bp, 132 bp, and 220 bp. products respectively. No PCR products were obtained during amplification with the negative control or non-target DNA templates from other bacterial species.

Conclusion. Designed primers can be used for the development of PCR system for the qualitative and quantitative detection of RSSC members.

Key words: PCR, *Ralstonia solanacearum*, RSSC, bacterial wilt.

Ralstonia solanacearum species complex (RSSC) is a diverse group of plant pathogens (*R. solanacearum, Ralstonia pseudosolanacearum, and Ralstonia syzygii*), which belongs to the family *Burkholderiaceae* [1, 2]. Members of RSSC are gram-negative soil-borne pathogens that cause devastating losses in the world agrarian economy due to lethal wilting in plants as a result of vascular dysfunction [3–5] in susceptible hosts [6–9]. RSSC causes bacterial wilt disease in over 450 different crop plant species covering more than 54 botanical families such as *Cucurbitaceae, Brassicaceae, Solanaceae, Fabaceae, Musaceae, Zingiberaceae*, and *Asteraceae* over the world [10–13].

Bacterial wilt is one of the most spread diseases of the potato. High-level expansion of the pathogen is associated with latently infected planting material. The disease is liable for causing substantial losses to the potato industry where it exists [9, 14–16]. In European Union (EU), RSSC was included in the EPPO A2 List of pests recommended for controlling and eradicating [2]. This actualizes necessity of regular epidemiological monitoring of RSSC. For routine practice, a detection assay should be rapid, specific, and quite sensitive. In addition, the methodology for the extensive monitoring should not be labor intensive and time-consuming. According

to the EU control directive, wide range of methodological approaches are acceptable for primary screening and monitoring, including serological techniques, the enzyme-linked immunosorbent assay, and molecular techniques such as PCR [17–20]. The latter is reliable and unequivocal method, which meets the majority of aforementioned requirements and provides high levels of confidence in identification, high sensitivity and specificity.

RSSC is highly heterogenic. Initially, *R. solanacearum* was divided into five races and six biovars based on the host plants and biochemical features. Currently, RSSC is assorted into four phylotypes based on the analysis of the ITS region [9, 19] and strains' geographic origins: Phylotype I (Asia) —*Ralstonia pseudosolanacearum*; Phylotype II (America) — *R. solanacearum*; Phylotype III (Africa and India Ocean) — *Ralstonia pseudosolanacearum*; Phylotype IV (Indonesia) — *Ralstonia syzygii* [9, 19, 20]. The most widely used PCR assays for quarantine purposes or to confirm the introduction of the pathogen to areas where it was not present are usually based on the amplification of ribosomal gene sequences (i.e., 16S or 16S-23S intergenic spacer region of the ribosomal DNA [rDNA]) with the use of species-specific RS primers for detection of all RSSC strains [21].

The objective of this study was to compare specificity of routinely used primer mix for PCR RSSC detection with newly developed pairs of species-specific primers for ease of use in a diagnostic laboratory.

Materials and Methods

Bacterial strains and growth conditions. The 14 strains from the Ukrainian National Collection of Microorganisms were kindly provided by Prof. V. Patyka (Department of Phytopathogenic Bacteria, D. K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, Kyiv). These reference strains, isolated from a variety of hosts and geographic locations, are representative causal agents of the bacterial diseases of solanaceous plants (are listed in Table 1.) Bacteria were grown at 28 °C on potato agar (potato 500g/L, NaCl 5g/L, agar 20g/L) for 24–48 h.

DNA preparation. Bacterial genomic DNA was extracted using the Agrosorb NK kit (LLC Agrogen Novo, Ukraine). The purity and quantity of isolated DNA were determined spectrophotometrically by absorbance measurement of A260/A280 using a spectrophotometer NanoDrop 1000 (Thermo Fisher Scientific, USA) and DNA concentration is estimated by measuring the absorbance at 260 nm.

PCR analysis. A pair of primers OLI-1/Y-2 was used for molecular identification of *Ralstonia solanacearum* strains [2, 22]. The conserved genomic regions of the 16S rRNA sequences of *R. solanacearum, R. pseudosolanacearum*, and *R. syzygii* were selected for the design additional primers for this study (Table 2).

Polymerase chain reaction was performed in a final volume of 10 µl, and reaction mixture consisted of 3.4 µl double-distilled water, 3.1 µl 2×PCR SuperMix (Bio-Helix, Taiwan) 1 µl primer mix (concentration 5 µM), 2.5 µl template DNA. Sterile double-distilled water was used as a negative control to test for the presence of contamination in PCR reagents.

PCR was carried out on a T100™ Thermal Cycler (Bio-Rad Laboratories Ltd., USA). After the initial denaturation step for 96 °C at 2 min, reactions of amplification were performed using 40 cycles. Cycles consisted of a 20 s denaturation at 94 °C, 20 s of primer annealing at 66 °C and 30 s extension at 72 °C, followed by a final step at 72 °C 10 min and cooling to 4 °C.

PCR-amplified fragments were electrophoresed in the horizontal 2% (m/v) agarose gel with ethidium bromide in SB (Sodium Borate or Sodium Boric Acid) buffer at 100 V for 1 hour.

Visualization of amplified fragments was conducted by UV light (260 nm) and photographed using Bio-Rad Gel DocTM XR+ gel documentation system (Bio-Rad Laboratories Ltd., USA).

Results and Discussion

The *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ATCC 11696 (ICMP 5712), also known as K60, is *R. solanacearum* type strain, which belongs to phylotype II, sequevar 7. K60 was isolated in 1953 from a Marglobe tomato (*Lycopersicon esculentum*) in Raleigh, North Carolina, USA, and has been used extensively for bacterial wilt research [23]. According to Seal et al. (1993) [22], the use of specific primer OLI1 together with the non-specific primer Y2 in PCR with an annealing temperature of 68 °C allows specific amplification of a single 287–288 bp product from the strain K60.

***Table 1*. Bacterial strains used in the study**

Species	Strain code
Ralstonia solanacearum (Smith 1896) Yabuuchi et al. 1992	B-1109T = ATCC 11696, CFPB 2047, ICMP 5712, ICPB PS256, LMG 2299, NCPPB 325 ← Mypac B.A., 9049 ← ICMP 5712 ← NCPPB 325
Ralstonia solanacearum (Smith 1896) Yabuuchi et al. 1992	B-1110 = ICMP 7859 ← Mypac B.A., 9081 ← ICMP 7859.
Clavibacter michiganensis subsp. *michiganensis*	Ac-1996
Clavibacter michiganensis subsp. *sepedonicus*	Ac-1995
Clavibacter michiganensis subsp. *sepedonicus*	Ac-1997
Pectobacterium carotovorum	B-1075
Pectobacterium atrosepticum	B-1084
Pectobacterium atrosepticum	B-1103
Pectobacterium carotovorum subsp. *carotovorum*	B-1097
Pectobacterium carotovorum subsp. *carotovorum*	B-1079
Pseudomonas syringae pv. *syringae*	B-1022
Pseudomonas syringae pv. *syringae*	B-1028
Xanthomonas campestris pv.*campestris*	B-1070
Xanthomonas vesicatoria	B-1060

Table 2. **Primer sets used in this study**

Primer	Sequence (5′-3′)	Direction	Reference
OLI*	GGGGGTAGCTTGCTACCTGCC	Forward	Seal et al. (1993)
Y2	ACTCCTACGGGAGGCAGCAGTGGG	Reverse	Seal et al. (1993)
OLI160	CGGCCGCCTCCTATAGCATGA	Reverse	Designed in the study
OLI248	AGTCCCAGTGTGGCTGATCG	Reverse	Designed in the study
Rs28	TGGCGAACGGGTGAGTAATA	Forward	Designed in the study
Rs193	GGCCTTTACCCCACCAACTA	Reverse	Designed in the study

* All primers were synthesized by Metabion, Germany.

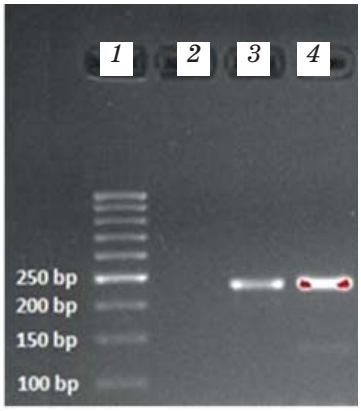

Fig. 1. **PCR result using OLI1/Y2 specific primers:**
1 — DNA size markers, Step Ladder 50 bp; *2* — negative control;
3 — *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ICMP 5712;
4 — *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ICMP 7859.

The *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ICMP 7859 was isolated by I. Herrera in 1974 from a potato (*Solanum tuberosum* L.) in Morropon, Piura, Peru, and was deposited in ICPM in 1983. Data concerning PCR identification of this isolate is absent. ICMP 5712 and ICMP 7859 both belong to the same biovar I [24].

According to Seal et al. [22], Y2 primer was designed by Young et al. (1991) [25] to amplify a partial segment (corresponding to positions 4&337 of *Escherichia coli* 16S rRNA) of most DNAs encoding 16s rRNA. And, for specific PCR amplification of specimens containing DNA of the RSSC members, the non-specific primer Y2 is used together with a specific primer OLI1 primer. In our experiments, OLI1/Y2 pair of primers produced the unexpectedly short 250-bp-long-fragment instead of expected 288-bp-long-fragment from both *R. solanacearum* strains: ICMP 5712 and ICMP 7859 (Fig. 1). Relative humidity- or/and temperature-induced transition from B-DNA to A-DNA could be one of the reasons of obtaining PCR product which is shorter than expected, since in a B-A transition, the long and narrow B-duplex is converted to the under wound and compact A-DNA structure [26–28].

Next, we tested specificity of newly constructed primers for the amplification of parts of consensus sequence for RSSC [22]. The homology of the suggested primer sequences was analyzed using the Basic Local Alignment Search Tool (BLAST) to avoid primer-template mismatches and to increase their specificity to the wide range of isolates. The primers were designed to obtain relatively short PCR products that would distinctly differ in length after the electrophoresis. Reverse primers were constructed (OLI-160 and OLI-248) on the basis of forward primer, proposed by Seal et al. (1993) [22]. Additionally, a pair of primers Rs-28 and Rs-193, which formed the amplification product within 166 bp was selected. For the testing of newly created primers specificity, we used two abovementioned strains of *R. solanacearum*, as well as 12 strains of other phytopathogenic bacteria.

Seven pairs of constructed primers were combined with the expected amplification products of 160, 248, 193, 166, 132, 220, and 260 bp correspondingly. The pairs of primers OLI/OLI-160, OLI/OLI-248 and OLI/Rs193 did not give any products. As one can see on the Fig. 2, four of seven tested pairs of primers provided only single specific fragments for bacterial strains of *Ralstonia solanacearum* of expected length. Namely, pairs of newly created primers Rs-28(F)/Rs-193(R), Rs-28(F)/OLI-160(R), Rs28(F)/OLI248(R) produced 166 bp, 132 bp, and 220 bp. products respectively. Rs28(F)/Y2(R) pair produced 260-bp-long-fragment. No PCR products were obtained during amplification with the negative control or non-target DNA templates from other bacterial species.

Since the development of primers was carried out in the perspective of creating a PCR test system for the detection and quantitative analysis of RSSC in potato biomaterial, phytopathogens affecting both solanaceous and some non-solanaceous plants were chosen for the specificity testing (Table 1).

For *Clavibacter michiganensis* subsp. *michiganensis*, tomato is the most important host. In addition, other solanaceous plants including potato are susceptible upon natural infection and/or artificial inoculation [29].

Clavibacter sepedonicus is not widely distributed in the areas where potato evolved. Nevertheless, *C. sepedonicus* has the tendency to exist asymptomatically as latent infections in potato. Involuntary spread of the bacterium to new places of production occurs with the movement of latently infected seed tubers used for planting [30].

Pectobacterium spp. are pectolitic necrotrophic pathogens responsible for a blackleg and soft rot disease of potatoes in many areas. These quarantine and threatening pathogens cause considerable economic and yield losses, both in field production and storage [31].

Xanthomonas vesicatoria causes bacterial spot disease in tomato and pepper, as well as in various other *Solanaceae*, and can be detected on fruits of potato [32]. The remaining two phytopathogens did not affect solanaceous plant including potato: *Pseudomonas syringae* affects woody and herbaceous host plants [33] and *Xanthomonas campestris* pv. *campestris* causes black rot of crucifers [34]. Such a wide range of phytopathogenic bacteria of different host specificity allowed us to thoroughly test the specificity of newly created primers.

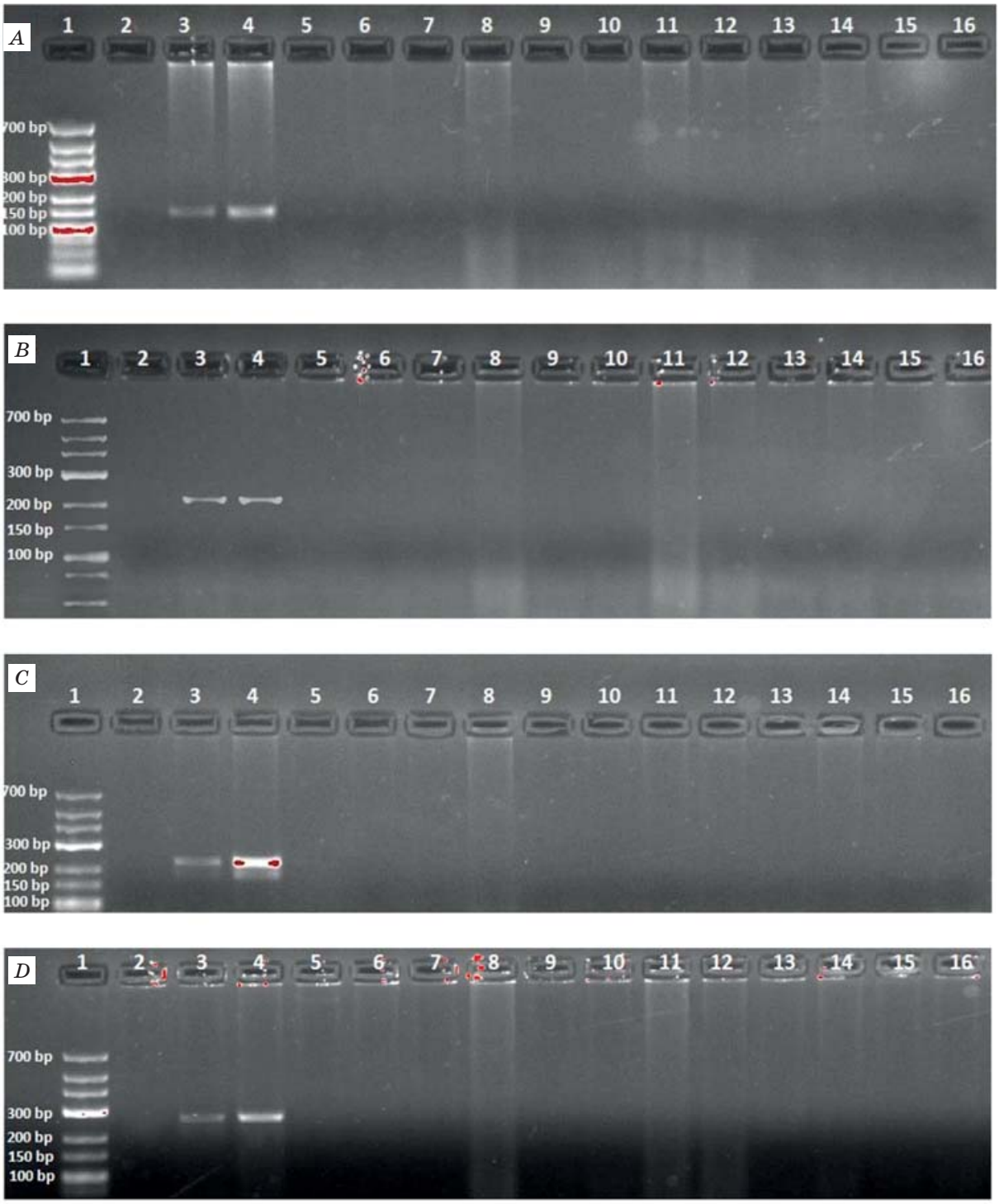

Fig. 2. **PCR results using Rs-28/Rs-193 (*A*), Rs-28/OLI-160 (*B*), Rs28/ OLI248 (*C*) and Rs28/Y2 (*D*) pairs of primers:**
1 — DNA size markers, Step Ladder 50 bp; 2 — negative control; 3 — *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ICMP 5712; 4 — *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1992 ICMP 7859; 5 — *Clavibacter michiganensis* subsp. *michiganensis* (Ac-1996), 6 — *Clavibacter michiganensis* subsp. *sepedonicus* (Ac-1995), 7 — *Clavibacter michiganensis* subsp. *sepedonicus* (Ac-1997), 8 — *Pectobacterium carotovorum* (B-1075), 9 — *Pectobacterium atrosepticum* (B-1084), 10 — *Pectobacterium atrosepticum* (B-1103), 11 — *Pectobacterium carotovorum* subsp. *carotovorum* (1097), 12 — *Pectobacterium carotovorum* subsp. *carotovorum* (B-1079), 13 — *Pseudomonas syringae* pv. *syringae* (B-1022), 14 — *Pseudomonas syringae* pv. *syringae* (B-1028), 15 — *Xanthomonas campestris* pv.*campestris* (B-1070), 16 — *Xanthomonas vesicatoria* (B-1060).

Conclusion

Based on 16S RNA and OLI1/Y2 primer pairs, four primers specific for the RSSC were designed and tested for species specificity. Results of species specificity testing evidence that three pairs of designed primers, as well as constructed specific forward primer RS28 paired with routinely used non-specific Y2 reverse primer can be used for the development of PCR system for the qualitative and quantitative detection of RSSC members.

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Conflicts of Interest

Authors declare no conflict of interest.

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СТВОРЕННЯ СПЕЦИФІЧНИХ ПРАЙМЕРІВ ДЛЯ АНАЛІЗУ ГЕНА 16S РРНК У ВИЯВЛЕННІ ВИДОВОГО КОМПЛЕКСУ *Ralstonia solanacearum*

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Представники видового комплексу *Ralstonia solanacearum* (RSSC) є збудниками судинного в'янення більше ніж 450 видів сільськогосподарських культур, включаючи пасльонові рослини, такі як картопля, помідори, болгарський перець, баклажани тощо. Ці фітопатогени спричиняють серйозні втрати врожаю переважно пасльонових культур, які вирощують у регіонах світу з тропічним, субтропічним та помірним кліматом. Втрати врожаю коливаються від 80 до 100% для картоплі, до 91% для помідорів, 10–30% для тютюну, 33–90% для бананів, знижуючи продуктивність і врожайність зазначених культур. ПЛР є специфічним, чутливим та економічно ефективним методом для виявлення та ідентифікації представників RSSC.

Мета. Порівняння специфічності стандартно використовуваної суміші праймерів для виявлення представників RSSC методом ПЛР з парами новостворених видоспецифічних праймерів для зручності використання в діагностичній лабораторії.

Матеріали та методи. Консервативні геномні ділянки послідовностей 16S рРНК *R. solanacearum*, *R. pseudosolanacearum* і *R. syzygii* було відібрано для створення праймерів. Видоспецифічність новостворених праймерів перевіряли за допомогою ПЛР з використанням ДНК двох цільових і 12 нецільових штамів бактерій.

Результати. Три пари новостворених праймерів Rs-28(F)/Rs-193(R), Rs-28(F)/OLI-160(R), Rs28 (F)/OlI248(R) продукували специфічні фрагменти для штамів бактерій *Ralstonia solanacearum*: 166 пн, 132 пн і 220 пн. Жодних продуктів ПЛР не було отримано під час ампліфікації з негативним контролем або нецільовими матрицями ДНК з інших видів бактерій.

Висновок. Створені праймери можна використовувати для розроблення ПЛР тест-системи з метою якісного та кількісного виявлення представників RSSC.

Ключові слова: ПЛР, *Ralstonia solanacearum*, RSSC, бактерійне в'янення.

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PECULIARITIES OF THE GROWTH OF *Artemisia tilesii* Ledeb. "HAIRY" ROOTS WITH DIFFERENT FOREIGN GENES

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Aim. To compare *Artemisia tilesii* "hairy" root lines with different transferred genes in terms of the relationship between the total content of flavonoids, the levels of antioxidant activity (AOA) and reducing power (RP), as well as the activity of phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), *rol*B and *rol*C genes.

Methods. We compared the root lines Nos. 10 and 16, obtained by transformation with the wild *Agrobacterium rhizogenes* strain A4, lines Nos. 2 and 4, obtained using *A. rhizogenes* carrying pSV124 vector with *ifn-α2b* and *npt*II genes, as well as the roots of non-transformed plants that were cultivated *in vitro*. The presence and activity of *rol*B, *rol*C, *PAL*, and *CHS* genes were determined by PCR and real-time PCR, respectively. The content of flavonoids, AOA and RP were determined by standard tests with $AlCl_3$, DPPH (2,2-diphenyl-1-picrylhydrazyl) and $K_3[Fe(CN)_6]$ accordingly.

Results. The content of flavonoids in most of the lines was higher than in the control, and correlated with AOA and RP. Roots No. 10 were characterized by the fastest growth, which coincided with higher activity of *rol*B and *rol*C genes. The activities of *PAL* and *CHS* in "hairy" roots were lower than those in non-transformed ones.

Conclusions. Root lines carrying only *rol*B and *rol*C and lines with additional *ifn-α2b* and *npt*II genes had similar ranges of flavonoids concentration, AOA and RP levels that exceeded those in the control. The dependence of the root growth rate, and lack of the dependence of the flavonoids content with the activity of the *rol* genes were demonstrated. *PAL* activity inversely correlated with flavonoids content in all experimental lines, which may be the result of overproduction of compounds in transgenic roots.

Key words: *Artemisia tilesii* Ledeb., *Agrobacterium rhizogenes*, "hairy" roots, flavonoids, *rol* genes, *PAL* and *CHS* genes, real-time PCR, antioxidant activity.

Medicinal plants have long been used both in traditional medicine and in the pharmaceutical industry. This is due to the fact that they synthesize a number of biologically active compounds that can be used to treat human diseases. Among medicinal plants, the genus *Artemisia*, or wormwood, is quite famous. Many species belong to this genus. In particular, "Plants of the World Online", an online database published by the Royal Botanic Gardens, counts about 500 species of *Artemisia*, of which *A. annua* L. has recently become the most famous. It has been established that plants of this species are capable of synthesizing artemisinin, a compound with antimalarial and antitumor properties [1, 2]. The Nobel Prize was awarded to Chinese researcher Tu Youyou in 2015 for such studies of annual wormwood (https://www.nobelprize.org/prizes/medicine/2015/tu/lecture/). Recently, publications have appeared on the possibility of using wormwood to inhibit the replication of the dangerous SARS-CoV-2 virus [3].

Among the numerous representatives of the genus *Artemisia*, the little-studied plants *A. tilesii* Ledeb., which have a very limited natural area of growth [4], should be singled out. It is known that they are used by the population of Alaska, on the territory of which they grow, for the treatment of various diseases. Since this plant material is scarcely available, genetic transformation and establishing of "hairy" root cultures can be a way to obtain biologically active compounds from plants of this species. Such roots can be maintained *in vitro* for decades, they are able to grow in bioreactor conditions on a nutrient medium that does not contain high-value components [5]. In addition, "hairy" roots synthesize the same compounds that the mother plants contain. It should be noted that due to the transfer of the *rol* genes of *A. rhizogenes* in the process of transformation, the content of valuable compounds can be significantly increased [6–8]. Such features make "hairy" root cultures of medicinal plants a valuable source of biologically active compounds [9, 10]. This is especially attractive for those plants that are rare, endangered, or not readily available.

It was determined that the bacterial *rol*B gene is a powerful inducer of the secondary metabolism of plants, affecting the activity of the native genes of "hairy" roots [6, 11–13]. For example, increased expression of this gene positively correlated with increased isochorismate synthase gene and with anthraquinone production [14]. In this study, the effect of the *rol*B gene exceeded the similar effect of other *A. rhizogenes* genes, namely *rol*A and *rol*C. The transfer of *rol*B and *rol*C genes to *A. annua* plants led to an increase in the content of artemisinin, artesunate, and dihydroartemisinin in transgenic plants [15]. In addition, an increase in the activity of genes involved in the biosynthesis of artemisinin was observed in these roots.

Previously, we obtained the "hairy" roots of *A. tilesii* and analyzed the accumulation of flavonoids and the level of antioxidant activity [16]. Significant fluctuations of those parameters were found in different lines, which is probably due to the non-determined site of incorporation of transferred genes into the plant genome. At the same time, it is of great interest to find out the features of correlation between the activity of the transferred *rol* genes of *A. rhizogenes* and the synthesis of flavonoids in the lines transformed by the A4 wild strain of *A. rhizogenes* and the same strain that additionally carried a plasmid with *npt*II and *ifn*-α*2b* genes. The present work was aimed at comparing such "hairy" root lines regarding the presence of an interrelation between the total content of flavonoids and the level of antioxidant activity, as well as the activity of the plant's own genes — phenylalanine ammonia-lyase (*PAL*) and chalcone synthase (*CHS*), and the *rol*B and *rol*C genes of *A. rhizogenes*.

Materials and Methods

Plant material

Four *A. tilesii* "hairy" root lines from the collection of the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine were used as the plant material for the study [17]. We obtained two of them (Nos. 10 and 16) by the transformation using *A. rhizogenes* A4 wild strain. The other two (Nos. 2 and 4) — using *A. rhizogenes* carrying pCB124 vector with the human interferon-α2b gene *ifn*-α*2b* and the selective neomycin phosphotransferase *npt*II gene. The roots were grown at a temperature of +24 °C for two weeks on the solidified nutrient Murashige and Skoog medium (Duchefa Biochemie) with halved macrosalt content (1/2 MS) and the addition of sucrose at a concentration of 20 g/l. For research, the roots were separated from the nutrient medium and washed with deionized water.

PCR analysis

Total plant DNA for polymerase chain reaction (PCR) was isolated according to the protocol [18], using the CTAB method. The following pairs of primers were used: 5′-CCTGAATGAACTCCAGGACGAGGCA-3′ and 5′-GCTCTAGATCCAGAGTCCCGCTCAGAAG-3′ (*npt*II, 622 bp, 65 °C); 5′-TTGATGCTCCTGGCACAG-3′ and 5′-TTCTGCTCTGACAACCTC-3′ (*ifn*-α*2b*, 396 bp, 60 °C); 5′-ATGGATCCCAAATTGCTATTCCTTCCACGA-3′ and 5′-TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3′ (*rol*B, 592 bp, 56 °C); 5′-TGGAGGATGTGACAAGCAGC-3′ and 5′-ATGCCTCACCAACTCACCAGG-3′ (*rol*C, 473 bp, 56 °C). The reaction was carried out on a Mastercycler personal 5332 amplifier (Eppendorf). PCR products were analyzed by electrophoresis in 1.5% agarose gel in a Tris-borate buffer system.

Real-time qPCR analysis

Total RNA was isolated from "hairy" root samples (~200 mg) of transformed and control

Table 1. **Primer sequences of the genes used for qPCR amplification**

Gene name	Primer sequences, 5′–3′	Accession number in the Gene bank or reference
β-actin	Forward: ATCAGCAATACCAGGGAACATAGT Reverse: AGGTGCCCTGAGGTCTTGTTCC	EU531837
chalcone synthase (*CHS*)	Forward: AGGCTAACAGAGGAGGGTA Reverse: CCAATTTACCGGCTTTCT	GQ468548
phenylalanine ammonia-lyase (*PAL*)	Forward: ACACTCGGTTAGCTATTGCTGCAA Reverse: CCATGGCGATTTCTGCACCT	JF806362
*rol*B	Forward: CTCACTCCAGCATGGAGCCA Reverse: ATTGTGTGGTGCCGCAAGCTA	[16]
*rol*C	Forward: TGGAGGATGTGACAAGCAGC Reverse: ATGCCTCACCAACTCACCAGG	[16]

A. tilesii cultures according to the high-throughput phenol-based method described in the paper [19]. DNase treatment of each RNA sample was performed in a 20 µl reaction mixture containing 3 µg total RNA, 2 U RNase-free DNase I (Thermo Fisher Scientific), 20 U RiboLock™ RNase inhibitor (Thermo Fisher Scientific), 1× Reaction Buffer (Thermo Fisher Scientific), adjusted to the final volume with DEPC-treated Milli-Q water. The reaction was carried out at 37 °C for 30 min using an Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific) and stopped by adding 2 µl of 25 µM EDTA, followed by incubation at 65 °C for 10 min. Reverse transcription (RT) was performed in a reaction mix containing 10 µl DNase-treated RNA sample, 40 U Maxima Reverse Transcriptase (Thermo Fisher Scientific), 20 U RiboLock™ RNase inhibitor (Thermo Fisher Scientific), 5 µM oligo(dT)$_{18}$ primer (Metabion, Germany), 1 µM dNTP (Thermo Fisher Scientific), 1× Reaction Buffer (Thermo Fisher Scientific), adjusted to a final volume of 20 µl with DEPC-treated Milli-Q water. The reaction was performed at 55 °C for 30 min in a thermocycler and stopped by incubation at 85 °C for 5 min.

Gene expression analysis was performed in 20 µl qPCR mix using HOT FIREPol → → EvaGreen → qPCR Mix Plus (no ROX; Solis BioDyne, Estonia) according to the manufacturer's protocol. The reaction mixture included gene-specific forward and reverse primers (0.5 mM each), and 2 µl RT mixture prepared above. The primer sets used for amplification of flavonoid biosynthetic genes and real-time qPCR conditions are given in Table 1. Three amplification reactions per sample were performed to obtain the average expression levels of the genes and internal standards. The relative expression levels of the genes of interest were calculated according to Pfaffl's equation [20].

Total flavonoids content

Determination of the content of flavonoids was carried out according to the Pekal and Pyrzynska method with modifications [21]. To prepare the extracts, the roots were separated from the medium, washed with deionized water, dried using filter paper, weighed 0.3 g each and homogenized in 3 ml of 70% ethanol. The homogenate was centrifuged in an Eppendorf Centrifuge 5415 C at 15 000 g for 10 min. The reaction mixture contained 0.25 ml of extract supernatant, 1 ml of deionized water, 0.075 ml of 5% $NaNO_2$ solution. After standing for 5 min, 0.075 ml of 10% $AlCl_3$ solution was added and held for another 5 min. Then 0.5 ml of 1M NaOH and 0.6 ml of deionized water were added. Absorption was determined at λ = 510 nm on a Fluorate-02-Panorama spectrofluorimeter. The calculation of the total content of flavonoids was carried out in the rutin equivalent (RE) according to the following formula, and converted to grams of fresh weight (FW):

$$C = (0.8842 \cdot OD) \cdot V / m, (R^2 = 0.9988)$$

where C — concentration of flavonoids in 1.0 g of fresh weight of plant material, mg RE/g FW; *OD* — optical density of the investigated solution, U; *V* — volume of ethanol used to prepare the extract; *m* — mass of plant material used for research.

Antioxidant activity (AOA)

The antioxidant activity of ethanol extracts of "hairy" roots was studied using the DPPH test according to the method described in [22]. The optical density of the solutions was

measured at a wavelength of λ = 515 nm on a Fluorate-02-Panorama spectrofluorimeter. The percentage of inhibition was calculated according to the following formula:

$$\%\ inhibition = (OD_1 - OD_2) / OD_1 \cdot 100,$$

where OD_1 — optical density of the DPPH solution, U; OD_2 — optical density of the of the reaction mixture after carrying out the reaction with DPPH, U.

The effective concentration (EC_{50}) was calculated as the fresh weight of the root (mg FW) required to scavenge 50% of DPPH in the reaction with the radical.

Reducing power (RP)

Determination of the ability of root extracts to reduce iron ions Fe^{3+} to Fe^{2+} was proceeded according to the method of Zhao *et al.* [23] with modifications [16]. The reaction mixture contained: 0.312 ml of 0.2 M phosphate buffer (pH 6.6); 0.312 ml of 1% potassium hexacyanoferrate(III) and ethanol root extract, the concentration of which was successively reduced. The cuvettes were incubated in a water bath at 50 °C for 30 min. After that, 0.312 ml of 10% trichloroacetic acid, 1.25 ml of deionized water and 0.25 ml of 0.1% iron(III) chloride were added to the reaction mixture. The optical density was measured at a wavelength of λ = 700 nm on a Fluorate-02-Panorama spectrofluorimeter. Reducing power was characterized by the effective concentration parameter ($EC_{0.5}$), which corresponded to the roots weight (mg FW) required to obtain OD = 0.5.

Weight gain

The terminal parts of the roots (growth points) with a length of about 10 mm were separated, weighed and cultivated on the surface of the agarized 1/2 MS medium at a temperature of +24 °C. After two weeks, the grown roots were separated from the agar, washed with distilled water, dried using filter paper and weighed on a Sartorius balance with a standard deviation of ± 0.005 g. Weight gain was determined as the difference between the final and initial weights in terms of one growth point.

Data analysis

All analyzes were performed in triplicate. Results were calculated in Microsoft Excel and presented as mean ± SD. The data were analyzed for statistical significance using ANOVA followed by Tukey HSD test using R software version 4.0.4. The difference between mean values were considered statistically significant at $P < 0.05$.

Results and Discussion

PCR analysis of four *A. tilesii* lines selected for research confirmed the presence of *rol*B and *rol*C genes in all the lines (Nos. 2, 4, 10, 16) and the absence of those genes in the control roots (C). This result affirms the successful transformation of all four lines (Fig. 1, *A* and *B*) and presence of the genes during 8 years of *in vitro* subcultivation.

Two lines (Nos. 10 and 16) were obtained by *A. rhizogenes*-mediated transformation with the A4 wild strain. The others two (Nos. 2 and 4) using *A. rhizogenes*, carrying the pCB124 vector with the human interferon-α2b gene and the selective neomycin phosphotransferase II gene, i.e., which additionally had *ifn-α2b* and *npt*II genes. PCR analysis confirmed the presence of these genes in lines Nos. 2 and 4 (Fig. 1, *C*). These genes were not detected in control roots.

It was established that the weight gain of the root lines varied significantly (Fig. 2, 3). Line No. 10 was characterized by the fastest growth (weight gain was 2.31 ± 0.22 g). Weight gain of the roots Nos. 2, 16, and 4 was 1.52 ± 0.09 g, 1.51 ± 0.19 g, and 1.77 ± 0.13 g, respectively.

The content of flavonoids in lines Nos. 2, 10 and 16 was higher than that in control roots (2.31 ± 0.42 mg RE/g FW) and varied from 3.86 ± 0.22 mg RE/g FW in line No. 16 to 9.47 ± 1.97 mg RE/g FW in line No. 2 (Fig. 4). It should be noted that the content of those compounds in line No.4 was 2.57 ± 0.28 mg RE / g FW and did not differ from the control.

Such an increase in the content of flavonoids in extracts from most of "hairy" root lines compared to non-transformed plants can be explained by an increase in the level of synthesis of secondary metabolites after the transfer of *rol* genes, as they are known to be activators of metabolism in plants. Significant variability in the parameters between the individual lines is probably caused by the non-determined incorporation of transferred genes into the plant genome. It is also important to indicate that no differences in flavonoids accumulation were observed between the two groups of lines. "Hairy" roots obtained as a result of transformation with a wild strain of agrobacteria (lines Nos. 10 and 16) and those containing *ifn-α2b* and *npt*II genes (lines Nos. 2 and 4) had approximately the same range of flavonoids concentration. Therefore, it can be concluded that these additional genes do not affect the biosynthesis of polyphenolic compounds.

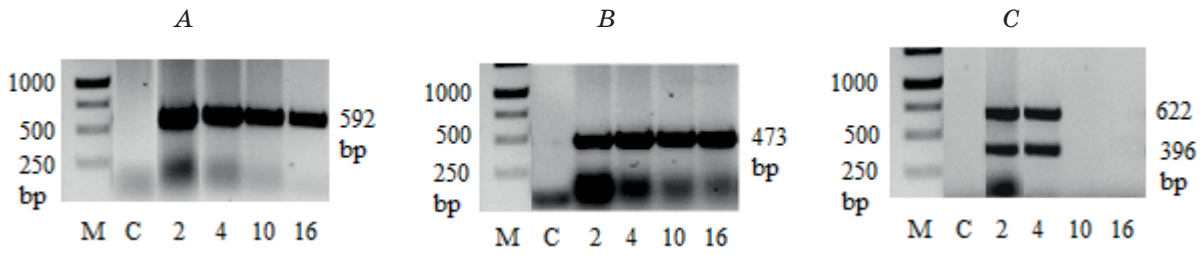

Fig. 1. **Electrophoregrams of DNA amplification products in samples of *A. tilesii* with gene-specific primers to *rol*B (*A*), *rol*C (*B*) and *npt*II i *ifn*-α*2b* (*C*)**
Tracks: M — marker GeneRuler 1 kb DNA Ladder (Thermo Scientific); c — DNA of control plant; 2, 4, 10, 16 — "hairy" root lines Nos. 2, 4, 10, 16.

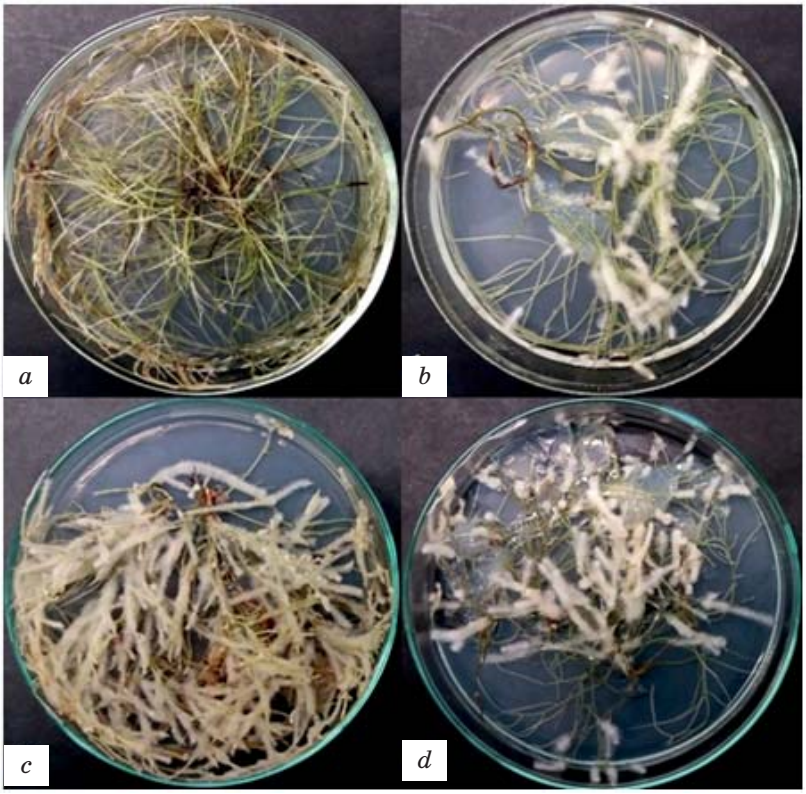

Fig. 2. **"Hairy" roots of *Artemisia tilesii:* lines No. 2 (*a*), No. 4 (*b*), No. 10 (*c*), No. 16 (*d*) after two weeks of *in vitro* cultivation:**
lines Nos. 2 and 4 were transformed with wild *A. rhizogenes* strain A4; lines Nos.10 and 16 were transformed with *A. rhizogenes* carrying *ifn*-α*2b* and *npt*II genes.

Similarly, the antioxidant activity and reducing power of the studied lines were higher than those of the control roots. For example, in the line No. 2 EC_{50} was 1.92 mg FW, and $EC_{0.5}$ was equal to 1.38 mg FW. It was 4.68 and 3.42 times higher, respectively, than the values in non-transformed roots. Both EC_{50} and $EC_{0.5}$ varied between different lines, but it was found out that in all lines, the higher was the flavonoid content, the lower was the EC_{50} and $EC_{0.5}$ values, and thus the higher was the antioxidant activity and reducing power. Such a correlation can be explained by the antioxidant nature of polyphenolic compounds. Polyphenols have the ideal chemical structure to activate the scavenging of free radicals, and they are more effective antioxidants than tocopherols and ascorbates. The antioxidant properties of polyphenols derive from their high reactivity as hydrogen or electron donors. The ability of flavonoids to change the kinetics of peroxidation by changing the order of lipid packing and reducing membrane fluidity is another mechanism of the effect as the radical scavengers [24, 25]. Similar to the total content of flavonoids in the studied

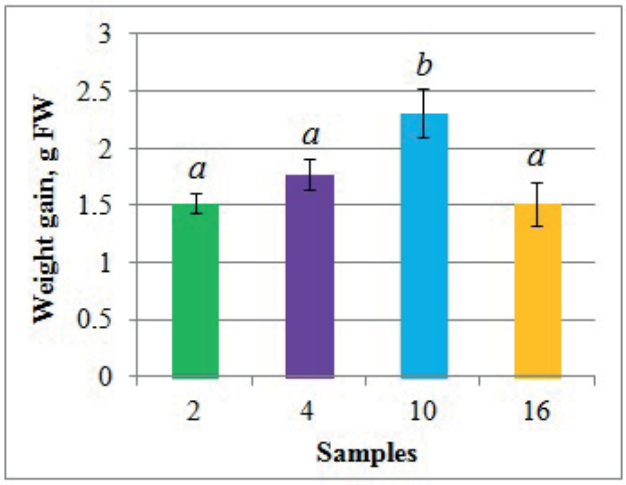

Fig. 3. **Weight gain of the *A. tilesii* "hairy" roots:**
columns 2 and 4 — lines Nos. 2 and 4 transformed with wild *A. rhizogenes* strain A4; 10 and 16 — lines Nos. 10 and 16 transformed with *A. rhizogenes* carrying *ifn*-α*2b* and *npt*II genes.
Error bars with different small letters denote significant differences in values among four samples at $P < 0.05$

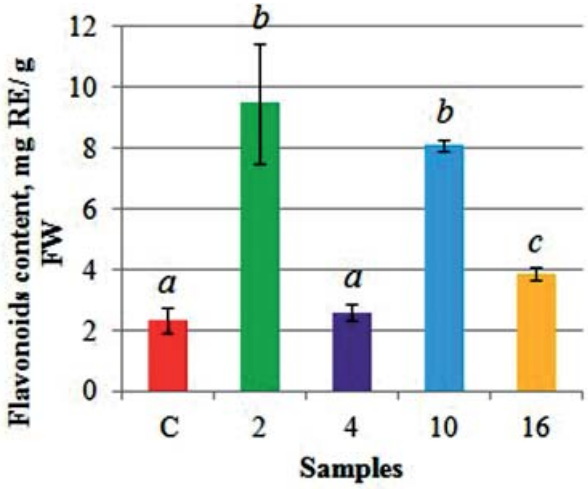

Fig. 4. **Total content of flavonoids in "hairy" and control (C) roots of *A. tilesii:***
columns 2 and 4 — lines Nos. 2 and 4 transformed with wild *A. rhizogenes* strain A4; 10 and 16 — lines Nos. 10 and 16 transformed with *A. rhizogenes* carrying *ifn*-α*2b* and *npt*II genes.
Error bars with different small letters denote significant differences in values among four samples at $P < 0.05$

plant extracts, no differences were found in the range of AOA and RP between the roots obtained as a result of transformation with *A. rhizogenes* wild strain and the bacterial strain carrying additional genes (*ifn*-α*2b* and *npt*II).

The results of real-time PCR showed that the activity of the transferred *rol*B (Fig., 5 *a*) and *rol*C (Fig. 5, *b*) genes was different in the studied lines, regardless of agrobacteria strain they were transformed with. It should be noted that the activity of both genes in line No. 10 was higher than in the other three lines. The relative activity of *rol*B was 3.05–9.34 times higher, and *rol*C was 2.46–5.38 times higher than in the other lines. The relationship between the *rol* genes activity and the weight gain of *A. tilesii* "hairy" roots was studied. Indeed, line No. 10 had the fastest growth rate compared to lines Nos. 2 and 16. The same result was obtained when analyzing the relative activity of the *rol*B gene. This can be explained by the mechanisms of *rol* genes action when they are transferred into the plant genome. Specific root formation in plants is the main function of *rol*B gene. It has a decisive influence on the formation of both lateral and adventive roots. Thus, the difference in growth rate among "hairy" root lines may be due to the difference in the *rol*B gene expression level [26].

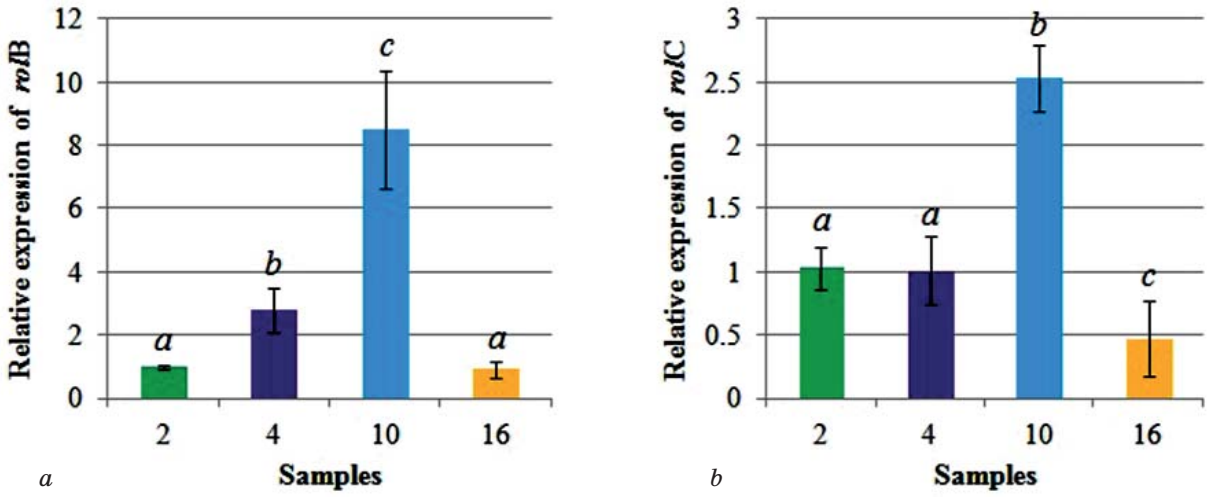

Fig. 5. **Relative activity of *rol*B (*a*) and *rol*C (*b*) genes in the "hairy" roots of *A. tilesii* after 2 weeks of cultivation:**
columns 2 and 4 — lines Nos. 2 and 4 transformed with wild *A. rhizogenes* strain A4; 10 and 16 — lines Nos. 10 and 16 transformed with *A. rhizogenes* carrying *ifn*-α*2b* and *npt*II genes.
Error bars with different small letters denote significant differences in values among four samples at $P < 0.05$

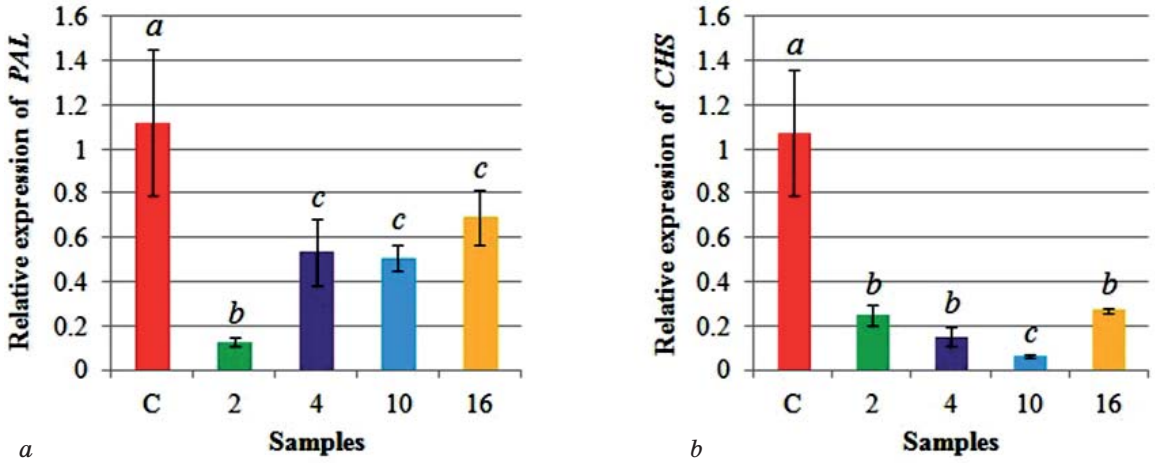

Fig. 6. **Relative activity of *PAL* (*a*) and *CHS* (*b*) genes in the "hairy" roots of *A. tilesii* after 2 weeks of cultivation:**
columns 2 and 4 — lines Nos. 2 and 4 transformed with wild *A. rhizogenes* strain A4; 10 and 16 — lines Nos. 10 and 16 transformed with *A. rhizogenes* carrying *ifn*-α*2b* and *npt*II genes
Error bars with different small letters denote significant differences in values among four samples at $P < 0.05$

Although the results of this study confirmed the influence of the activity of the transferred *rol* genes on the growth rate of the formed *A. tilesii* "hairy" roots, no interrelation between their activity and the synthesis of flavonoids was observed. This may be due to the fact that the function of *rol* genes as activators of secondary metabolism in plants is determined not by the activity of these transferred genes, but by the fact of their presence, and the site of their incorporation into the plant genome.

The study of the relative activity of phenylalanine ammonia-lyase (*PAL*) and chalcone synthase (*CHS*) showed that the level of activity of these genes in the root lines was different, but was lower than in the control. Since these genes are involved in the biosynthesis of polyphenolic compounds such as flavonoids and phenylpropanoids in plants, their activity may influence flavonoids content. As it turned out, in all experimental samples higher flavonoids content corresponded to lower *PAL* activity.

For example, the content of flavonoids in the line No. 2 was the highest among all samples and was 4.10 times higher than in control non-transformed plants (Fig. 6, *a*). Similarly, *PAL* activity in the line No. 2 was 8.62 times lower than in the control. Such an inverse correlation was observed in all experimental lines. This can be explained by the reverse inhibition of the *PAL* gene by the high content of the synthesized product.

The level of *CHS* gene activity in control plants was 4.01–16.72 times higher than in *A. tilesii* "hairy" roots (Fig. 6, *b*). This result could also be the outcome of reverse inhibition of the chalcone synthase gene involved in the synthesis of polyphenols. As in all previous studies, the dependence between the activity of the phenylalanine ammonia-lyase and chalcone synthase genes and the presence of either *rol* genes only or together with *ifn*-α*2b* and *npt*II genes among the two groups of "hairy" roots was not detected.

Conclutions

In this paper, for the first time, the potential dependence of the activities of bacterial *rol* genes and the plant's own *PAL* and *CHS* genes present in the "hairy" roots, as well as their growth rate, total flavonoid content, antioxidant activity and reducing power on the presence of additional genes in the transformed roots, namely *npt*II and *ifn-2b*, were studied. The study confirmed the effect of *rol* genes as activators of secondary metabolism, in particular, on increasing the content of flavonoids, AOA and RP in most of the experimental lines of *A. tilesii* wormwood compared to the control plants. However, no interdependence of the relative activity of *rol* genes and flavonoids synthesis was found. Nevertheless, according to the results of the study, it was established that the activity of the *rol*B gene affects the growth rate of "hairy" roots. In particular, in all root lines the greater weight gain was observed in samples with higher activity of *rol*B gene. In addition, the level of phenylalanine ammonia-lyase activity was inversely correlated with the content of flavonoids in all experimental lines: the higher was the content, the lower was the *PAL* activity. The relative activity of both *PAL* and *CHS* was highest in the control roots. Such a result may indicate the presence of reverse inhibition of these two genes by the products of biosynthesis in which they are involved, due to the high content of flavonoids in the transformed plants.

Of special interest was the confirmation or refutation of whether the additional presence of transferred *npt*II and *ifn-2b* genes in "hairy" roots affects all the above-mentioned indicators. As a result of the study, it was found that there were no fundamental differences in the ranges of flavonoids content, AOA and RP, as well as the relative activities of the *rol*B, *rol*C, *PAL* and *CHS* genes between the two groups of "hairy" roots.

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ОСОБЛИВОСТІ БІОСИНТЕЗУ ФЛАВОНОЇДІВ У «БОРОДАТИХ» КОРЕНЯХ *Artemisia tilesii* Ledeb. З РІЗНИМИ ПЕРЕНЕСЕНИМИ ГЕНАМИ

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Мета. Порівняти лінії «бородатих» коренів з різним набором перенесених генів стосовно наявності взаємозв'язку між загальним вмістом флавоноїдів, рівнем антиоксидантної (АОА) та відновлювальної (ВА) активності, а також активності власних генів рослин — фенілаланін-амоній-ліази (*PAL*) та халконсинтази (*CHS*) і генів *rol*B і *rol*C *A. rhizogenes*.

Методи. Лінії коренів №10 і 16 отримано з використанням *A. rhizogenes* A4, № 2 та 4 — з використанням *A. rhizogenes* з вектором pCB124 (*ifn*-α*2b* та *npt*II гени).

Результати. Вміст флавоноїдів у всіх лініях був вищим за контроль і корелював з АОА та ВА активністю. Корені №10 відзначались найшвидшим ростом, що збігалося з високою активністю *rol*B та *rol*C генів. Активність *PAL* та *CHS* у «бородатих» коренях була нижчою за активність у нетрансформованих коренях.

Висновки. Лінії коренів, до яких було перенесено лише *rol*B та *rol*C, і лінії з додатковими генами *ifn*-α*2b* та *npt*II мали близький діапазон концентрації флавоноїдів, рівнів АОА та ВА, які перевищували ці показники в контролі. Встановлено залежність швидкості росту коренів та відсутність залежності вмісту флавоноїдів від активності *rol* генів. Активність *PAL* обернено співвідносилась із вмістом флавоноїдів в усіх дослідних лініях, що може бути результатом надпродукції сполук у трансгенних коренях.

Ключові слова: *Artemisia tilesii* Ledeb., *Agrobacterium rhizogenes*–опосередкована трансформація, «бородаті» корені, флавоноїди, *rol* гени, антиоксидантна активність.

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INFLUENCE OF FIBRIN D AND DD FRAGMENTS ON FIBRINOGEN AND FIBRINOGEN FRAGMENT X POLYMERIZATION INITIATED BY THROMBIN OR ANCISTRON

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Aim. Study of the role of the complex between the αC region and the BβN domain in the initial stages of fibrin polymerization has been investigated.

Materials and Methods. Method of turbidimetry to study the influence of fibrinogen fragments D and DD on the polymerization and methods of isolation, purification, fragmentation for fibrinogen, monomer and cross-linked fibrin, fibrinogen X-fragment, Glu -plasminogen were used.

Results. It was shown that fragment DD completely inhibited polymerization process in all the systems examined ("Fg + Thr", "Fg + Anc H", "X + Thr", "X + Anc H"). Fragment D inhibited fibrin polymerization at all stages in the system "Fg + Thr", but in the system "Fg + Anc H" it almost did not influence fibrin polymerization. In the both systems "X + Thr" and "X + Anc H" fragment D weakly inhibited the self-assembly of fibrin molecules into protofibrils, but accelerated the process of lateral association in the second system.

Conclusions. The data obtained indicated that the complex between the αC region and the BβN domain of fibrin desA, on the initial stage of polymerization supported the rate of self-assembling and lateral association of fibrin desA protofibrils, protecting the oligomers against the depolymerizing influence of fibrinogen.

Key words: fibrinogen, fibrin, fragment D, fragment DD, fragment X, BβN-domain-αC-region complex.

Fibrinogen is a multidomain protein that is involved in a series of biological processes in an organism: blood clotting and fibrinolysis, wound healing, angiogenesis, inflammation and metastasis.

The polymerization of fibrin is a two-stage process. On the first, enzymatic, stage, thrombin transforms fibrinogen into fibrin desA by cleaving fibrinopeptides A (FpA) from the N-ends of Aα-chains. The removal of fibrinopeptides A exposes the "A"-sites of polymerization ("knobs") complementary to polymerization sites "a" ("holes") located in the γ-module of the D region. Both polymerization sites ("A" and "a") are located on the "facial" surface of fibrin desA molecule [1]. It is considered that interaction between polymerization sites "A" and "a" is a "driving power" for fibrin polymerization — the self-assembling of fibrin molecules into the fibril network of the clot [2]. In the protofibrils having formed out of fibrin desA, the removal of fibrinopeptides B (FpB) by thrombin accelerates significantly and polymerization cites "B" ("B"-"knobs") on the N-ends of β-chains are exposed. The appearing of the sites "B" causes the acceleration of protofibril lateral association due to interaction between the sites "B"-"b" [3].

After fibrinopeptide A removal of fibrinogen molecule and forming of contacts "A"-"a" and "C"-"c" in fibrin desA oligomers, the connection between fibrinopeptide A and the αC-region and the connection between αC regions in fibrin desA molecule are broken down [4, 5]. According to X-ray structural analysis, the region 1–53 of the fibrinogen BβN domain is quite mobile [6]. However, after fibrinopeptide A removal and the DDE triad formation the two sites of the region — fibrinopeptide B (Bβ1–14) and Bβ28–36 — remain connected to the αC region [5, 7. 8]. As result, the domain BβN and the αC domain form a new temporary structure with limited mobility. It includes the region 37–54 of the fibrin Bβ chain, which has the length of about 50 Å and binds this temporary structure to the E module of the molecule. The structure can be supposed to be located between neighboring DDE triads in the protofibril. This assumption was confirmed by the data of the X-ray structural analysis of chicken fibrinogen [9].

An assumption was made that an important role in providing the high rate of protofibril formation is also played by BβN-domain-αC-region complexes, which remain on the surface of the molecule of fibrin desA [7, 8, 10]. The presence of the BβN-domain-αC-region complex at the very beginning of polymerization process, interactions "A"-"a" and D-D-long, the formation of the DDE triad altogether suggest an important role of the complex in fibrin polymerization process. These assumptions were confirmed by the comparative analysis of the polymerization processes of fibrinogen and, lacking for αC regions, fibrinogen fragment X both stimulated by thrombin and ancistron, and by the inhibition of these processes by fibrin fragments D and DD.

Materials and Methods

Fibrinogen was isolated from citrate human blood plasma in the presence of soybean trypsin inhibitor by salting out with Na_2SO_4 [11]. Glu-plasminogen was isolated from donor blood plasma by affinity chromatography using Lys-sepharose [12]. Ancistron — reptilase-like enzyme was isolated from venom of Agkistrodon halys halys (13). Fragment D was purified from the plasmin digest of noncross-linked fibrin by the method of ion-exchange chromatography on CM-Sephadex G-50 ("Pharmacia", Sweden) in accordance with [14]. D dimer was obtained from the plasmin digest of cross-linked fibrin by the aid of affine chromatography on fibrin-sepharose according to [15]. D dimer was dialyzed against 0.05 M ammonium acetate buffer pH 8.5 and freeze-dried.

Preparation of fibrinogen fragment X. Fragment X was isolated as described in [16] with some modifications. Fibrinogen dissolved in 0.02 M HEPES buffer pH 7.4, 0.15 M NaCl, 1.0 mM $CaCl_2$ was treated with plasmin in molar ratio of 1 mole of plasmin to 1000 mole of fibrinogen. Plasmin was formed immediately in the reaction medium from plasminogen activated by streptokinase in molar ratio of plasminogen to streptokinase as 10:1. The reaction was carried out during 20 min at 25 °C and was stopped by aprotinin in 20-fold molar surplus. The digest was passed through the Lys-Sepharose column, concentrated and applied on the Sephacryl S-300 column 3×100 cm, equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, containing 2 M NaCl, 100 mM 6-aminocaproic acid, 1 mM $CaCl_2$. The fractions containing fragment X_1 according to PAAGE were dialyzed against 0.02 M HEPES buffer pH 7.4 with 0.15 M NaCl, 0.005% NaN_3. The purity of the preparation was tested by ELISA with anti-αC and anti-BβN-domain monoclonal antibodies. The fragment X preparation was frozen and stored at –20 °C.

The effect of D and DD-dimer fragments on the fibrinogen polymerization, initiated by thrombin or ancistron, was studied with turbidimetry method using spectrophotometer SF 2000 at 350 nm (17, 18).

Results and Discussion

To clarify the role of the BβN-domain-C-region complex in fibrin polymerization process, we compared the rates of the polymerization of fibrin desA and desAB forms, which that either contained this complex or did not and the influence of polymerization inhibitors (fragments D and DD) on the separate stages of the polymerization process.

The BβN-domain-αC-region complex was present in the native fibrinogen molecule but was absent in fibrinogen fragment X, causing higher maximal clot turbidity level for the polymerization of fibrinogen in both thrombin (Thr) and ancistron H (Anc H) containing systems (Fig. 1.1, 1.2). In the system "Fg + Thr", fragment D inhibited significantly all the stages of fibrin polymerization: protofibrill self-assembling, lateral association of protofibrils and the reaching of the maximal

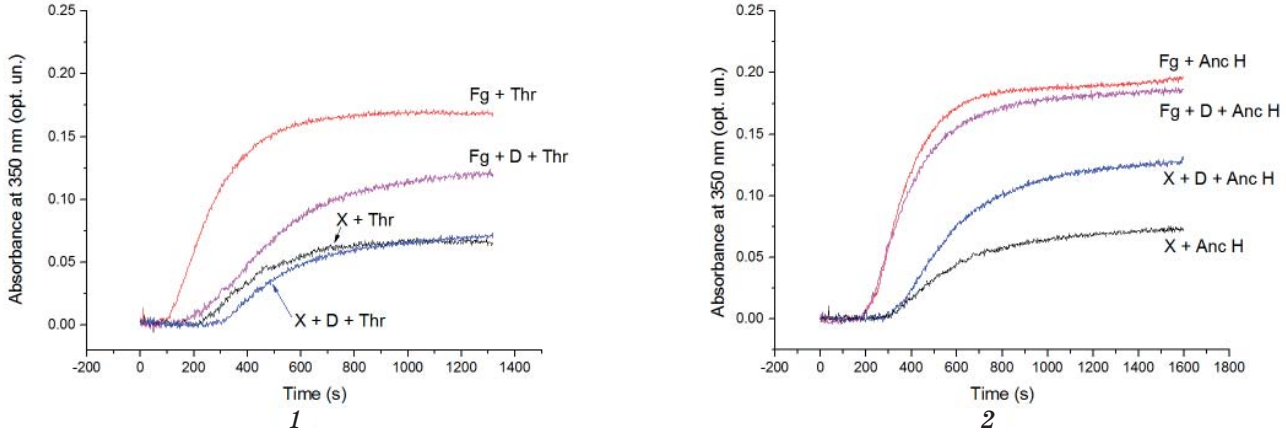

Fig. 1. **Influence of fragment D on polymerization of fibrinogen and fibrinogen fragment X initiated by thrombin (*1*) and ancistron (*2*) in reaction medium containing 0.02 M HEPES, pH7.4, 0.15 M NaCl and 1 mM $CaCl_2$**

Concentration of fibrinogen was 0.3 μM, fibrinogen fragment X — 0.3 μM, fibrin fragment D — 1.8 μM, thrombin and ancistron — 0.1 NIH/ml.

clot turbidity level (Fig. 1.1). Thrombin, having removed fibrinopeptide A, started to remove fibrinopeptide B after a short delay connected with the formation of protofibrils, which resulted in the degradation of the complex formed by the BβN domain and the αC region and thus the increasing of the accessibility of polymerization sites "A", "B", "C" for fragment D. In the system "Fg + Anc H", fragment D almost did not make an influence on fibrin polymerization process, because the complex of the BβN domain and the αC region remained in this system and protected polymerization sites A and C against inhibition by fragment D. Additionally, in the complex mentioned, the αC regions promoted the self-assembling of fibrin molecules into protofibrills and the lateral association of the last ones. In the system "X + Thr" (Fig. 1.1), fragment D weakly inhibited the self-assembling of fibrin molecules into protofibrils and did not influence the process of lateral association. In the fragment desA-X, the D region interacted with the E region more effectively than isolated fragment D, because this interaction carried out by two pairs of polymerization sites, "A"-"a" and "C"-"c", while the interaction with fragment D was due to one pair of polymerization sites. In the system "X + Anc H" (Fig. 1.2), while fibrinopeptide B in fragment X remained intact, fragment D weakly inhibited the self-assembly of fibrin molecules into protofibrils but accelerated the lateral association of protofibrils. This could be explained by the inclusion of fragment D into the structure of a clot, which was mediated by the presence of fibrinopeptide B and/or primary lateral association γC-γC contacts of D-fragments (1). The absence of αC region in fragment X and the desintegration of the complex of the BβN domain and the αC region in fibrin desA resulted in the deceleration of protofibril formation and lateral association, which indicated an important role of the complexes in these processes.

Fragment DD completely inhibited polymerization process in all the systems examined ("Fg + Thr", "Fg + Anc H", "X + Thr", "X + Anc H"), evidently, due to the presence of three pairs of polymerization sites (2a, 2b i 2c) localized in a single molecule and thus providing highly affine interaction between fragment DD and fibrin desA or fragment X on the very initial stages of polymerization. It is also essential that the binding of the DD fragment to the E region of the desA or desAB fibrin molecules blocks the end-to-end interaction of the D regions of the fibrin molecules at formation the basic structures of the protofibril — the DED triads.

In contrast to fragment D, which has three polymerization sites (a, b, c), fragment DD has a double number of these sites (2a, 2b, 2c) and thus completely blocks polymerization process of either fibrin desA or fibrin fragment X by competing effectively with the D regions of fibrin or fragment X for polymerization centres A, B and C.

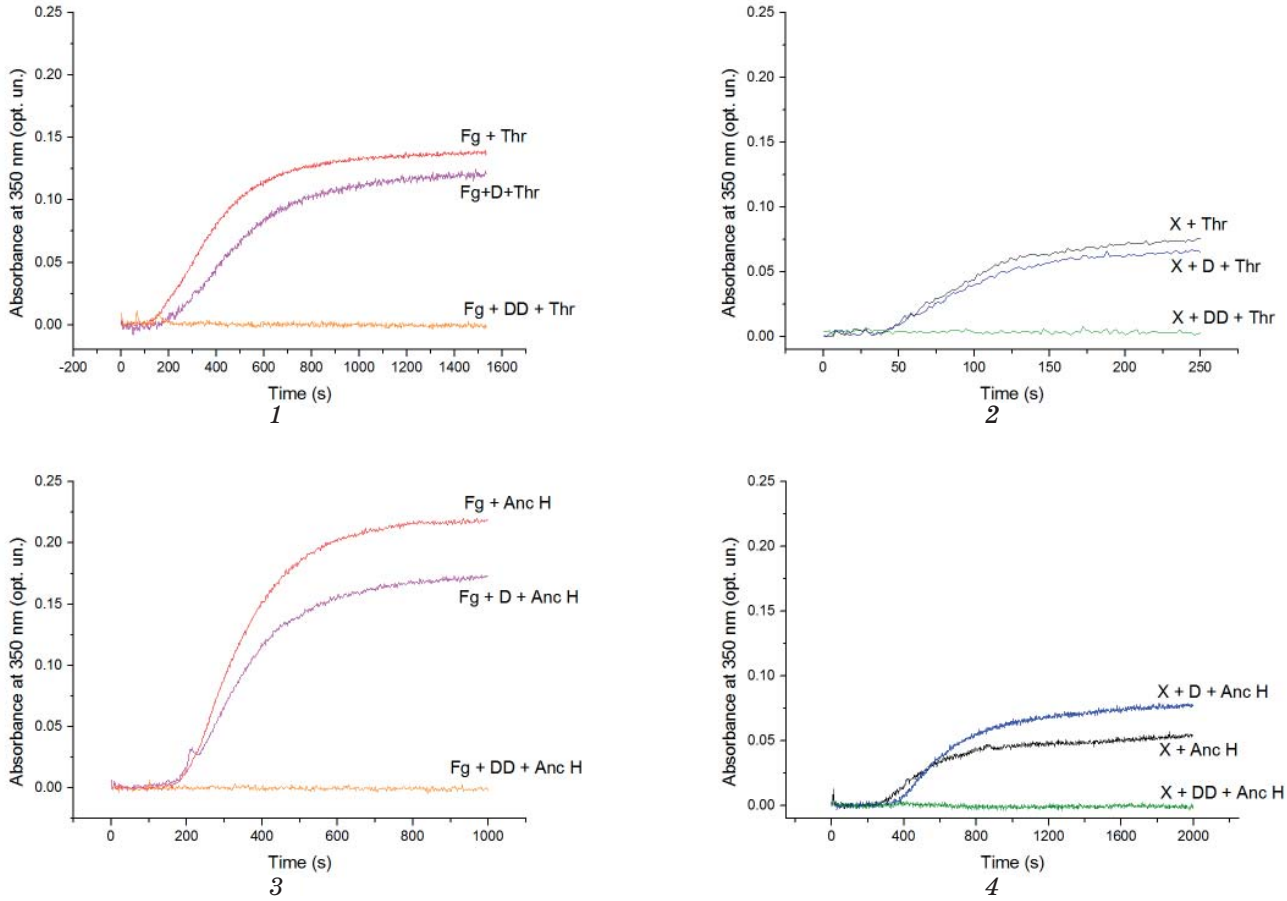

Fig. 2. **Comparison of the effects of fragments D and DD on initiated by thrombin and ancistron of fibrinogen polymerization**

Reaction medium contained 0.3 μM fibrinogen and fibrinogen fragment X — 0.3 μM, fibrin fragment D — 1.8 μM of each, in 0.02 M HEPES, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$. Concentration DD — 0.9 μM, thrombin and ancistron — 0.1 NIH/ml.

Conclusions

In fibrin desA, when, previously connected to the αC regions, fibrinopeptides A are removed, the complex between the BβN domain and the αC region becomes mobile and able to give access to polymerization sites on the molecule for other fibrin molecules or oligomers for further interactions. The mobility of the complex may be confirmed by the fact that fragment D inhibits polymerization of fibrin only partially and fragment DD does it completely in all the cases examined, which indicates the displacement of the complex by a more affine inhibitor. According to the evidence obtained, present only in fibrin desA molecules, the complex favours the interaction exactly of fibrin desA molecules with each other resulting in the formation of the DDE triad and promotes further protofibril formation and the lateral association of the protofibrils. Thus, the complex plays an important role in the growth of fibrin protofibrils as the result of the process of fibrin desA polymerization.

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Conflict of interest

Authors declare no conflict of interest.

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ВПЛИВ D І DD ФРАГМЕНТІВ ФІБРИНУ НА ПОЛІМЕРИЗАЦІЮ ФІБРИНОГЕНУ ТА ЙОГО ФРАГМЕНТА X, ЩО АКТИВУЮТЬСЯ ТРОМБІНОМ АБО АНЦИСТРОНОМ

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Мета. Досліджували роль комплексу між αC-регіоном та BβN-доменом, який формується після видалення FpA на молекулі фібрину desA, на початкових етапах полімеризації фібрину.

Методи. Для вивчення впливу D і DD фрагментів фібриногену на полімеризацію фібриногену і фрагменту фібриногену X було використано метод турбідиметрії, а також методи виділення, очищення та фрагментації фібриногену, мономерного та поперечно-прошитого фібрину, плазміногену.

Результати. Показано, що фрагмент DD повністю гальмував процес полімеризації у всіх досліджених системах («Fg + Thr», «Fg + Anc H», «X + Thr», «X + Anc H»). Фрагмент D інгібував полімеризацію фібрину на всіх стадіях у системі «Fg + Thr», але в системі «Fg + Anc H» майже не впливав на полімеризацію фібрину. В обох системах «X + Thr» і «X + Anc H» фрагмент D слабо пригнічував самозбирання молекул фібрину в протофібрили, але прискорював процес латеральної асоціації у другій системі.

Висновки. Отримані дані свідчать, що комплекс між αC-регіоном та BβN-доменом фібрину desA на початковій стадії полімеризації підтримує швидкість самозбирання та латеральної асоціації протофібрил фібрину desA, захищаючи його олігомери від деполімеризувального впливу фібриногену.

Ключові слова: фібриноген, фібрин, фрагмент DD, фрагмент X, комплекс BβN-домен-αC-регіон.

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Chelidonium majus WATER EXTRACT INITIATES PLATELET AGGREGATION AND INHIBITS FIBRIN POLYMERIZATION IN BLOOD PLASMA

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Chelidonium majus is a well-known source of biologically active compounds. Most of them are alkaloids, which are used in researches and for tradition medicine. In this study, we explored the influence of *C. majus* crude total extract onto blood coagulation system *in vitro*, primary and secondary hemostasis.

Aim. To study influence of *C. majus* extract onto blood coagulation process.

Methods. Turbidimetry of blood plasma, APTT test with chromogenic substrates, and platelet aggregation were used in the work.

Results. We demonstrated moderate stimulating effect of the extract on platelets (the rate of platelet aggregation increased up to 10%, followed by disaggregation). Extract also increased the rate of platelet aggregation stimulated by 12.5 mcM of ADP.

We observed the increase in the plasma clotting time in the presence of the extract, that corresponded to the 274, 411, 685, 1370 mcg of dry extract, from 70 s in control to 80, 90, 170 and 180 s, respectively, in turbidimetry test of plasma stimulated by APTT-reagent.

However, APTT test with the chromogenic substrate of thrombin (S2238) did not show significant influence of this plant's extract on thrombin activity.

Conclusions. Thus, we can conclude that anticoagulant activity of *Chelidonium majus* extract corresponded to the direct inhibition of fibrin polymerization.

Key words: *Chelidonium majus*, platelet activation, plasma coagulation, thrombin.

Chelidonium majus is known as celandine, common celandine, or greater celandine [1–3]. It belongs to the *Papaveraceae* family of dicotyledonous plants, and like its relative *Papaver somniferum*, comprises numerous biologically active compounds. This is a perennial herb, that grows in Europe and the Mediterranean, and is also common in America. Closely related species grow in Eurasia and Japan. Plants have a straight, branched stem 50–70 cm high.

Basal leaves are petiolate, deeply pinnate. They have three to five pairs of rounded or ovoid lobes. The upper leaves lobe is larger, usually, they are three-lobed. The leaves are glaucous below and green above. The upper leaves are sessile. The flowers are actinomorphic, golden yellow, with a characteristic formula $*K_2C_4A_\infty G_{(2)}$ [4]. The fruit is a polyspermous pod-shaped capsule.

There are a lot of biologically active compounds, such as alkaloids, in *Chelidonium majus* herb. This plant, its juice, herb and roots are used for science and for traditional medicine. In traditional medicine, it was used to improve eyesight and as sedative, antispasmodic and for curing bronchitis, whooping cough, asthma, jaundice, gallstones, and gallbladder pain (celandine)

[1]. Juice with latex is used to treat skin diseases and problems such as warts, ringworm and corns [1].

Also isoquinolin extracted from *Chelidonium majus* was active against some pathogenic bacteria and *Candida* [5].

It consists of more than 35 compounds [2, 3]. It comprises a lot of alkaloids, derivatives of benzophenanthridine: homohelidonine, chelidonine, chelerythrine, chelidocystatin, coptisine, sanguinarine, berberine, protopine, sparteine and another [1, 6, 7]. It consists of non-alkaloid compounds, such as ascorbic acid, carotene, saponins, bitters, latex and protein [8, 9].

Also, it consists of agarose-like polysaccharides, yellow or orange milky juice. The experience of using this plant in traditional medicine was not so successful because of cytotoxicity [10], which was shown in clinical trials [11].

The aim of the present work was to study the influence of *Celidonium majus* extract onto hemostasis, blood coagulation process. This work highlights the influence of *C. majus* total water extract on protein and cellular hemostasis *in vitro*.

Material and Methods

We prepared *Chelidonium* herb in May during its flowering, dried in the shadow at 25 °C, and packed in cardboard boxes for the further use. The dried herb was cut into small pieces with scissors and steamed in a tris-HCl buffer, pH 7.4, and then was heated to 100 °C for 12 hours in a foam box. We routinely used 4 g of cut herb per 200 ml of buffer. Also, 1 ml of extract was dried on the small Petry dish with weight control before and after filling and drying, which helped to calculate the mass of dry extract per 1 ml. Blood plasma and platelet-rich plasma samples were obtained from blood of healthy donors. Volunteers signed informed consent prior to the blood sampling according to the Helsinki declaration. Platelet aggregation was studied using Solar AP2110 Aggregometer (Belarus). Blood plasma clotting was measured using Solar CGL-2410 Coagulometer (Belarus). Chromogenic substrate assay was performed using Multiscan EX (Thermo, USA).

Fibrin formation in blood plasma under the action of APTT-reagent was determined using POP spectrophotometer (Optizen, Korea). The scattering of light was measured at 350 nm. The following reagents were consecutively added to the 10 mm spectrophotometric cuvette: blood plasma (100 mcl) was mixed with 100 mcl of APTT-reagent and incubated 3 minutes at 37 °C. Then 0.05 M tris-HCl buffer, pH 7.4, containing 0.15 M NaCl (400–600 mcl) and 100 mcl 0.025 M $CaCl_2$ were added to start the reaction. Analysis of the turbidity curves were performed using a specialized computer program.

Results and Discussion

In our study we used volumes of *Chelidonium majus* extract (0, 20, 30, 50, 100 mcl) that corresponded to the 0, 274, 370.5, 617.5, 1235 mcg of dry extract, that was characterized in [12].

For the determining of the effects of *Chelidonium majus* extract on platelets we performed aggregometry studies. Platelets in the platelet-rich blood plasma (250 mcl) were mixed with 25 mcl of 0.025 M of $CaCl_2$ at 37 °C and 20 mcl (274 mcg) of the extract or equivalent volume of 0.05 M tris-HCl buffer at 25 °C. We demonstrated moderate stimulating effect of the extract on platelets (the rate of platelet aggregation increased by up to 10%, followed by disaggregation). The extract also increased the rate of platelet aggregation stimulated by 12.5 mcM of ADP [12].

To study the effect of the extract of proteins on the blood coagulation system we applied activated partial thromboplastin time (APTT) test. It was performed in accordance with the following procedure. 0.1 ml of blood plasma was mixed with an equal volume of APTT-reagent (Siemens, Germany) and incubated for 3 min at 37 °C. Then the extract of *Chelidonium majus* or equal volume of 0.05 M tris-HCl buffer, pH 7.4, was added. After that the coagulation was initiated by adding of 0.1 ml of 0.025 M $CaCl_2$ solution. As a result, we observed the increase in the plasma clotting time in the presence of 0, 20, 30, 50, 100 mcl of the extract (that corresponded to the 0, 274, 370.5, 617.5, 1235 mcg of dry extract) from 70 s in control to 80, 90, 170 and 180 s, respectively (Fig. 1).

The inhibition of blood clotting could be explained by the direct action on the fibrin polymerization, or by the suppression of the clotting cascade factors activities. To clarify the reasons of the observed effect we measured the thrombin activity in the APTT-activated blood plasma using thrombin-specific chromogenic substrate S2238 H-D-Phe-Pip-Arg-pNA at 405 nm. The analysis was performed in 0.05 M Tris-HCl buffer, pH 7.4, at 37 °C. Chromogenic substrate was

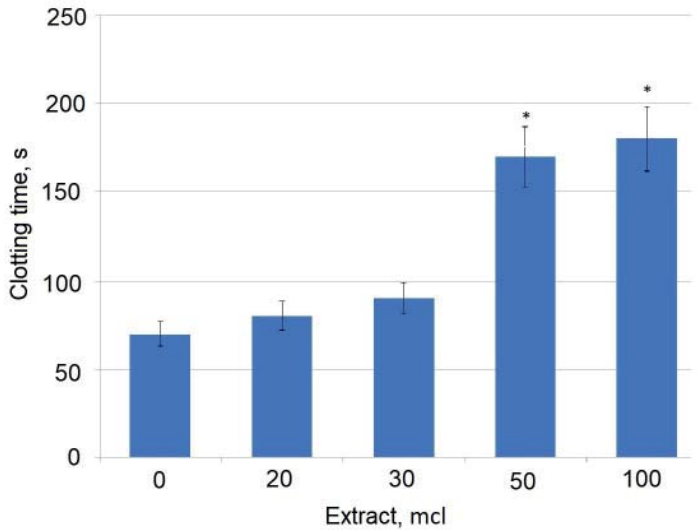

Fig. 1. **Time of blood plasma clotting induced by APTT-reagent in the presence of *Chelidonium majus* extract:**
(0, 20, 30, 50, 100 mcl of extract corresponded to the 0, 274, 370.5, 617.5, 1235 mcg of dry extract).
APTT — activated partial thromboplastin time. * $P \leq 0.05$.

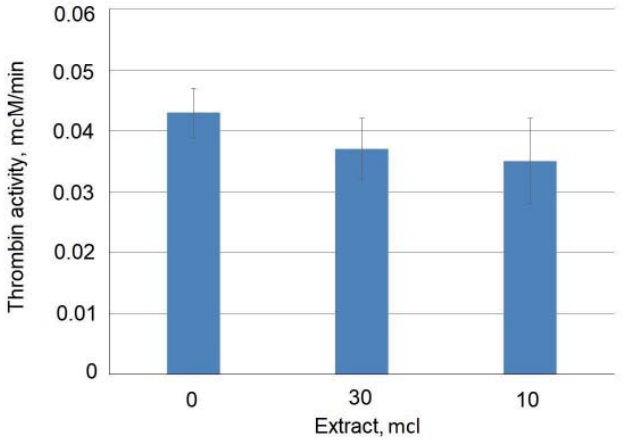

Fig. 2. **Activity of thrombin generated in blood plasma under the action of APTT-reagent in the presence of** Chelidonium majus **extract:**
(10, 30 mcl corresponded to 123.5, 370.5 mcg of dry extract). APTT — activated partial thromboplastin time.

taken in the final concentration 30 mcM. The tested amount of *Chelidonium majus* extract ranged from 10 to 30 mcl that corresponded to the 123.5, 274, 370.5 mcg of dry extract. The experiment did not show any inhibition or facilitation of thrombin activity in the presence of studied extract (Fig. 2).

This finding allowed us to conclude that *Chelidonium majus* extract compounds did not act on the clotting cascade factors, and the overall anticoagulant effect of the extract can be connected to the direct inhibition of fibrin polymerization.

To prove this hypothesis, we performed a turbidity study of the fibrin formation in the presence of *Chelidonium majus* extract. The tested amount of *Chelidonium majus* extract ranged from 30 to 100 mcl that corresponded to the 123.5 and 1235 mcg of dry extract. We demonstrated that fibrin polymerization was significantly inhibited by the studied extract, which was direct evidence of the action of *Chelidonium majus* extract on the fibrin polymerization (Fig. 3).

In this study, we showed the opposite influence of *Chelidonium majus* water extract on the factors of clotting cascade and on the platelets. The same result was obtained in the series of experiments: studied substance inhibited the clotting and slightly activated

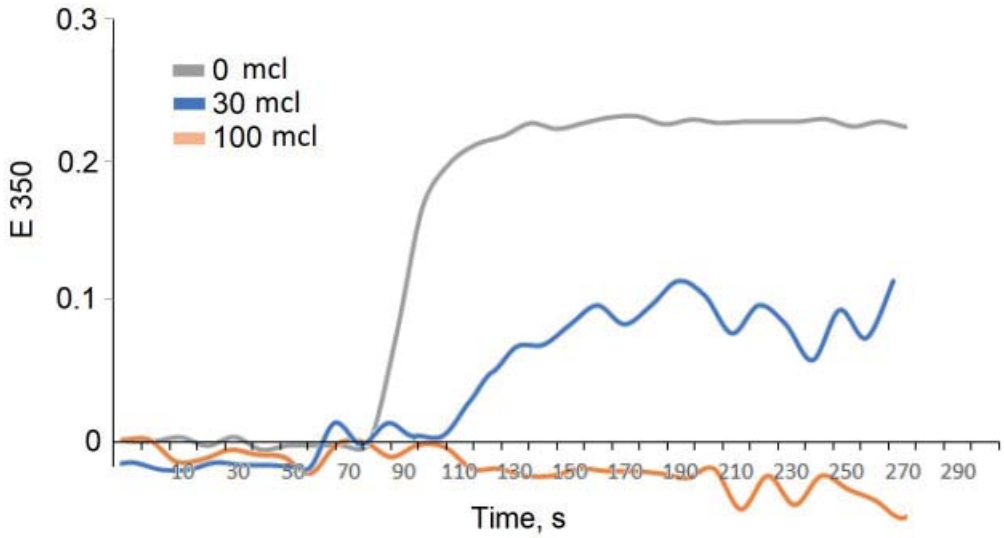

Fig. 3. **Turbidity study of blood plasma activated by APTT-reagent in the presence of *Chelidonium majus* extract:**
(10, 30 mcl corresponded to 123.5, 370.5 mcg of dry extract). APTT — activated partial thromboplastin time. Typical curves for 3 independent experiments are presented.

platelets. Platelets were moderately activated, but clot formation in the plasma was inhibited for a long period. We did not study these effects in intact fresh blood. There were difficulties in studying fibrin formation in turbidimetry methods in spectrophotometer cuvette, because of random oscillation of basal level of light scattering. This phenomenon may occur because of latex particles presented in the extract.

It is important to underline that the study was performed with *in vitro* isolated platelets or with platelet poor plasma components of the hemostasis system. This is a reduced model of the blood coagulation system, so the effects of pharmacokinetics and pharmacodynamics on the whole organism were not studied. Also, in this brief screening study, the goal of identification and isolation of active compounds was not performed. We suggest that some compounds of the extract may directly inhibit fibrin polymerization because there is no influence of the extract on thrombin in our experiments with a chromogenic substrate.

Also, it was shown that berberine, which is found in this plant, can activate platelets [12, 13] and cause their apoptosis [13]. It can also affect gene expression [14].

It is well-known that pure berberine is hydrophobic and did not form water solution. To make it soluble, the authors of [14] used a carrier — fullerene C_{60} to immobilize berberine on its surface.

However, berberine from *Chelidonium majus* was shown to be water soluble. This alkaloid is carried by some proteins like CmMLP1 [6], which makes it well soluble in water. Also, it can react with HCl from the extraction buffer, forming more soluble salt. Hydrochloride of berberine, obtained by treating this alkaloid by HCl-containing buffer, is sparingly soluble in water and biochemically active compound [15, 16].

Conclusions

Thus, we can conclude that the anticoagulant activity of *Chelidonium majus* extract possibly corresponded to the direct inhibition of fibrin polymerization. The ability of the water extract of *Chelidonium majus* to activate platelets was demonstrated for the first time. We can suggest, that this effect may be caused by berberine. However, this effect was rather moderate. We presume that the studied effect on plasma was rather nonspecific, however this question needs further exploration.

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Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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ВОДНИЙ ЕКСТРАКТ *Chelidonium majus* ІНІЦІЮЄ АГРЕГАЦІЮ ТРОМБОЦИТІВ І ПРИГНІЧУЄ ПОЛІМЕРИЗАЦІЮ ФІБРИНУ В ПЛАЗМІ КРОВІ

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Chelidonium majus (Чистотіл звичайний або великий) є добре відомим джерелом біологічно активних сполук, більшість з яких є алкалоїдами, що їх використовують у науці та традиційній медицині. Ми досліджували вплив водного екстракту *Chelidonium majus* на систему зсідання крові *in vitro*, первинний та вторинний гемостаз.

Мета. Вивчити вплив екстракту *Chelidonium majus* на процес згортання крові.

Методи. Турбідиметрія плазми крові, АЧТЧ-тест з використанням хромогенних субстратів та агрегація тромбоцитів.

Результати. Продемонстровано помірну стимулювальну дію екстракту на тромбоцити (швидкість агрегації тромбоцитів становила до 10 % з подальшою дезагрегацією). Екстракт також підвищував швидкість агрегації тромбоцитів, стимульовану 12,5 мкМ ADP.

В турбідиметричному тесті з використанням активації згортання АЧТЧ-реагентом спостерігали збільшення часу зсідання плазми крові за присутності екстракту в кількості, що відповідала 1370, 685, 411, 274 мкг сухого екстракту з 70 с в контролі до 170, 90, 80 і 180 с відповідно.

Водночас, тест АЧТЧ із хромогенним субстратом тромбіну (S2238) не показав істотного впливу екстракту цієї рослини на активність тромбіну.

Висновки. Таким чином, антикоагулянтна активність екстракту *Chelidonium majus* полягала у безпосередньому пригніченні полімеризації фібрину.

Ключові слова: *Chelidonium majus*, активація тромбоцитів, коагуляція плазми, тромбін.

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CHEMICAL MUTAGENESIS OF THE LYSINE-PRODUCING STRAIN *Brevibacterium* sp. IMV B-7447

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The aim of the work was to obtain a producer strain with increased lysine accumulation using the chemical mutagenesis method.

Methods. To achieve the goal, we used the method of treating the lysine-producing strain with the chemical mutagen N-methyl-N-nitro-N-nitrosoguanidine, cultivating the resulting clone and determining the accumulation of lysine in flasks and a bioreactor.

Results. The optimal concentrations and duration of mutagen action for the production of mutant microorganisms were found. Clones with the maximum lysine accumulation were selected. Mutagenesis was carried out consecutively three times. As a result, lysine-producing strain *Brevibacterium* sp. IMV B-7796 auxotrophic regarding leucine and threonine with maximum accumulation of the target amino acid was obtained.

Conclusions. The lysine producer *Brevibacterium* sp. IMV B-7796 was obtained, which produced 65.0 g/dm^3 of lysine in a bioreactor under conditions of periodic cultivation with feeding. The *Brevibacterium* sp. IMV B-7796 strain was proposed as a basis for the creation of a genetically modified strain with increased accumulation of lysine for further use in industrial lysine technology.

Key words: *Brevibacterium* sp., lysine, chemical mutagenesis, producer strain.

L-lysine is one of the essential amino acids, which is mainly used as feed additive. Traditional fodder crops such as corn, wheat or barley are low in lysine. The addition of 0.5% by weight of L-lysine increases the feed quality in the same way as the addition of 20% soybean meal [1]. The production of lysine globally was 2,200,000 tons per year as of 2014 [2]. As a feed additive, only L-form of lysine is effective, which is produced mainly by microbiological synthesis. In the microbiological production of α-amino acids and, in particular, lysine [3], the producer strains of *Brevibacterium* sp. (synonym of *C. glutamicum* [1]) are used.

Brevibacterium sp. are gram-positive, asporogenic, non-pathogenic bacteria, which by their nature are usually not capable of metabolites oversynthesis [2]. To further increase producer's productivity, traditional selection or genetic engineering are used. One of the ways to increase the strain's productivity is the selection of auxotrophic producers capable of oversynthesis of amino acids. Most of industrial lysine-producing strains are auxotrophic mutants [4, 5]. With the help of mutagenesis and selection, producers with auxotrophies for various amino acids, vitamins and resistance to antimetabolites were obtained. Producers with such mutations demonstrated a gradual increase in productivity [6, 7]. N-methyl-N′-nitro-N-nitrosoguanidine (NTG or MNNG) is one of the effective mutagens that leads to a change in the microorganism's phenotype, in particular, it induces auxotrophy and resistance to antibiotics [8–10].

Materials and Methods

The change in lysine accumulation by the producer strain *Brevibacterium* sp. IMV B-7447 [11] from the "Collection of microorganisms strains and plant lines for food and agricultural biotechnology of the State Enterprice "Institute of Food Biotechnology and Genomics of the National Academy of Sciences of Ukraine" was studied for using a mutagen.

Cultivation conditions and mediums. The strain was grown in dipped meat-peptone agar enriched with glucose for 24 hours. Composition of enriched meat-peptone agar (MPAer.) (g/dm^3): nutrient broth — 23.0, glucose — 10.0, yeast extract — 5.0, agar — 30.0, distilled water, pH 7.0±0.1

For the selection of colonies and auxotrophic detection, a minimal medium (MM) with the following composition (g/dm^3) was used: glucose — 30.0, $(NH_4)_2SO_4$ — 10.0, KH_2PO_4 — 2.0, $MgSO_4$ — 0.4, agar — 30.0; with the antimetabolite S-(2-aminoethyl)-L-cysteine (AEC, thialisin) (Sigma, USA) or amino acids (leucine, isoleucine, threonine, homoserine, methionine).

As an inoculation medium, the following composition was used (g/dm^3): glucose — 70.0, corn extract (KE) — 30; KE hydrolysate — 40; chalk — 10. Subsequently, 10% (by volume) of the inoculum was added to the enzymatic medium. As an enzymatic medium, the following composition was used (g/dm^3): glucose — 120.0, KE — 35.0; salt hydrolysate KE — 45; $(NH_4)_2SO_4$ — 40; $MgSO_4$ — 0.5; KH_2PO_4 — 2.0 mg, biotin — 200 µg/ml, thiamine — 250 µg/ml, glucose, amino acids, and 2% dry sterile chalk were added after sterilization. Cultivation of bacteria was carried out for 72 h in a shaker-incubator "BIOSAN" ES-20 (Latvia) at a temperature of 30 °C and a speed of 240 rpm.

Cultivation of a lysine-producing strain in a bioreactor

Cultivation was carried out in a bioreactor "Sartorius Biostat B TWIN" (Germany) with a paddle stirrer and a flask volume of 10 dm^3. The inoculum (10% by volume) was introduced into the bioreactor to the enzymatic medium with the initial volume of the medium filling 30% of the bioreactor volume.

The inoculation medium composition (g/dm^3): molasses — 60, KE hydrolysate — 40, KE — 30, $CaCO_3$ — 10, tap water. A culture loop from slant agar grown for 24 h was added to the medium. One shoal was distributed into flasks (0.5 dm^3) containing 30 ml of inoculation medium. Cultivation was carried out in a shaker-incubator for 20–22 hours at a temperature of 30 °C and a speed of 250 rpm.

The enzymatic medium composition (g/dm^3): glucose — 120, KE hydrolysate — 45, KE — 35, $(NH_4)_2SO_4$ — 40; $MgSO_4$ — 0.5; KH_2PO_4 — 2.0, leucine — 0.7, biotin — 200 µg, thiamin — 250 µg, tap water. Glucose was added to the medium separately after sterilization. Biotin, thiamin and leucine were added to the sterile medium in the form of sterile solutions.

The carbohydrate nutrition composition: glucose, molasses, $MgSO_4$, $(NH_4)_2SO_4$, tap water. The growth nutrition composition: corn extract, KE hydrolysate, tap water, KH_2PO_4, $(NH_4)_2HPO_4$, solutions of biotin and thiamine. Ready feed in 2 dm^3 glass bottles (Simax, Czech Republic) was connected to the bioreactor. Ammonia water (25%) was used for maintain the pH, as a defoamer — "Propinol B400".

The inoculum was grown using orbital shaker-incubator for 24 hours, and 10% by volume was added to the bioreactor with the enzymatic medium (initial volume 3 dm^3). The optical density of the inoculum was 0.3 units, pH 5.6–5.8. Technological parameters of cultivation were as follows: stirrer revolutions — (700–800) rpm; temperature — 30 °C; pH 7.0; air supply — (1.0–1.2) vol/rpm.

Carbohydrate feeding was added depending on the drop in glucose level. Growth feeding was administered guided by cultivation parameters such as ECO_2 (concentration of carbon dioxide in the exhaust gas mixture) and pO_2 (oxygen concentration in the medium). pH was adjusted automatically with ammonia water. The defoamer was added when the foam amount in the bioreactor flask exceed the predetermined level.

Chemical mutagenesis. Bacterial suspension (cell titer 10^6–10^7) was prepared from daily shoal in sterile physiological solution. The suspension of the selected clones was kept in a shaker (220 min^{-1}) at a temperature of 30–32 °C for 5–30 min in a Tris-malate buffer (pH 6.0) containing 100, 200, 300, 400 and 500 µg/dm^3 NTG, respectively. Then the cells were washed in 0.1 M Tris-phosphate buffer with pH 7.2. The bacterial suspension obtained by the dilution method was sown in Petri dishes in the MC containing the lysine analog — S-(2-aminoethyl)-L-cysteine (AEC) at a concentration of 0.4 mg/dm^3. The number of surviving cells was determined by the number of brevibacteria strains colonies grown in MPAer.

Auxotrophy research was carried out according to the methodology [12], modified for the needs of bacterial producers and nutrient media. To determine the strains auxotrophy, a bacterial suspension was taken, which was prepared as follows: a two-day culture was selected from cultures of MPAer, and diluted in sterile physiological solution to a concentration of 1×10^5 colony-forming units (CFU)/dm^3, which corresponded to 0.5 units of optical density (OD). The obtained inoculum was transferred sterilely into: complete medium (MPAer), MM, MM with the investigated amino acid and MM with the investigated antimetabolite (MM+AEC), respectively. Auxotrophy was determined by the presence of bacterial growth on the selected medium.

The medium pH was determined using a pH-meter "pH-150" (RF). Determination of glucose concentration was carried out using Fehling's solutions according to the method [13]. The amount of ammonium nitrogen was determined according to the method [14]. OG was measured by a KFK-3 photoelectrocolorimeter (RF) in cuvettes with a size between the walls of d = 5 mm at a wavelength of 440 nm. The amount of amino acids was determined with the amino acid analyzer "AAA-400" (Ingos, Czech Republic). Cytological studies were performed using a microscope "Laboval4" (Carl Zeiss, Germany). Photographs were taken with a "Canon PowerShot A640" camera (Japan).

Statistical data processing was done using Microsoft Excel. All experiments were performed in three replicates. The difference between two mean values was considered significant at $P < 0.05$.

Results and Discussion

One of the ways to obtain high-performance lysine producers is to obtain regulatory mutants, which are selected by insensitivity of their homoserine dehydrogenase enzyme to threonine. S-(2-aminoethyl)-L-cysteine (thialysine) was used as a selective agent. The resulting mutants were resistant to AEC and had two regulatory mutations that disrupted the retroinhibition of both homoserine dehydrogenase and aspartate kinase. Mutation in the gene coding aspartate kinase synthesis resulted in the loss of sensitivity to the reciprocal inhibition by lysine and threonine. In this case, the maximum accumulation of threonine and lysine occurred [15]. The tendency to increase the rate of lysine accumulation in mutants was associated with inhibition of homoserine dehydrogenase and homoserine kinase activity, as well as insensitivity of aspartate kinase to retroinhibition [16].

Only those cells in which the mechanism of negative regulation of amino acid biosynthesis was disrupted, and which synthesized an excess of the target amino acid, survived and formed colonies on the minimal medium with the AEC antimetabolite; this served as a criterion for the selection of mutant clones.

The most efficient of chemical mutagens, NTG, creates alkylation of bases in the replication fork, and forms mutants with transitions, transversions, and deletions. Mutations are found in most lysine producers treated with NTG, that prevent inhibition of aspartate kinase activity through the feedback of the coordinated action of L-lysine and L-threonine (gene lysC) or reduce the activity of homoserine dehydrogenase and reduce the availability of L-threonine in cells and thus also reduce the activity of kinase (gene hom), which is important for the creation of genetically modified strains [9, 10].

Considering the above, we determined the effect of NTG on the cells bacterial suspension of the strain *Brevibacterium* sp. IMV B-7447 to increase the accumulation of lysine. The percentage of cells that survived was determined by the number of colonies that grew on MPAer with AEC. Cell survival under the influence of NTG varied depending on its concentration and duration of action. Within two minutes of NTG exposure at a concentration higher than 200 μg/dm^3, no living cells remained. The greatest mutagenic effect, in which cell survival ranged from 0.1 to 1%, was obtained using a concentration of NTG of 100 μg/dm^3 and an exposure time of 20 min (Fig. 1).

After cultivation of the obtained clones on MPAer medium, colonies of different colors (without pigments, yellow and pink) and sizes were obtained (Fig. 2), which were selected for further determination of lysine production.

After 72 hours cultivation on the enzymatic medium, the obtained clones were studied and analyzed. The lysine accumulation by the resulting colonies exceeded its accumulation by the original strain. Colonies with a pink color eventually returned (after 2–3 reseeding) to the original yellow color, which indicated the mutation instability. Further studies were carried out only for the colonies of yellow color according to the following scheme (Fig. 3).

NTG-1, NTG-2, NTG-3 clones were initially grown on the slant MPAer for 24 hours, then

a loop with the cells was transferred to the inoculation medium for cultivation. After 24 hours they were passed into the flasks with enzymatic medium to select the clone with the greatest lysine accumulation. Cultivation of clones in a glucose enzymatic medium was carried out under conditions of aeration and T = 31±1 °C for 72 h (Fig. 4).

All clones produced lysine, but NTG-3 had the maximum level of lysine accumulation (36.0 g/dm^3) after 72 h cultivation in flasks (medium with glucose).

Brevibacterium sp. NTG-3 clone obtained by chemical mutagenesis was an auxotroph for threonine and leucine, while the parent strain *Brevibacterium* sp. IMV B-7447 was an auxotroph for leucine and methionine.

The initial culture and the resulting clone were tested for sensitivity to antibiotics. Both strains have been shown to be susceptible to antibiotics such as azithromycin, ampicillin, ceftriaxone, benzylpenicillin, gentamicin, tetracycline, streptomycin, and kanamycin and resistant to chloramphenicol.

To establish the stability of the *Brevibacterium* sp. NTG-3 clone with a changed phenotype, the clone was passed (for two months with an interval of two weeks) on a solid and liquid medium, and the accumulation of lysine was determined. The production of lysine by the clone did not change during the passing and amounted to 36.0 — 36.2 g/dm^3. The *Brevibacterium* sp. NTG-3 culture was deposited in the Depository of the Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine as *Brevibacterium* sp. IMV B-7796.

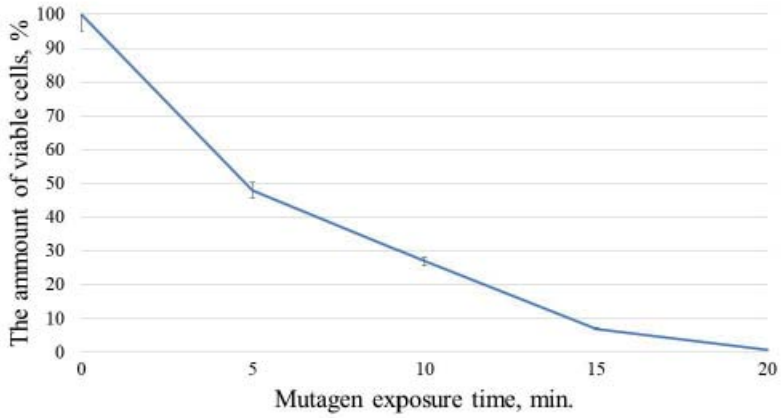

Fig. 1. **Cell survival of *Brevibacterium* sp. IMV B-7447 under the NTG influence at a concentration of 100 μg/dm^3**

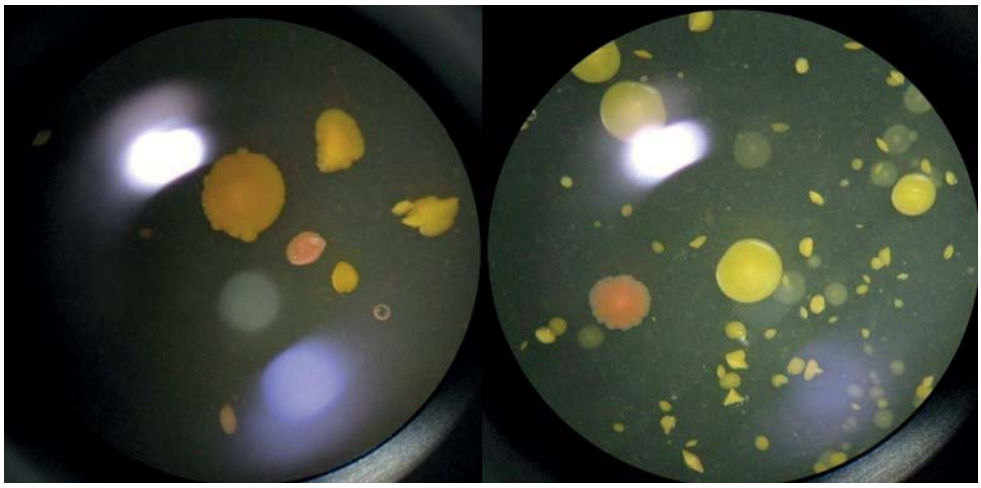

Fig. 2. **Colonies on MPAer medium after treatment with NTG mutant strain *Brevibacterium* sp. IMV B-7447**

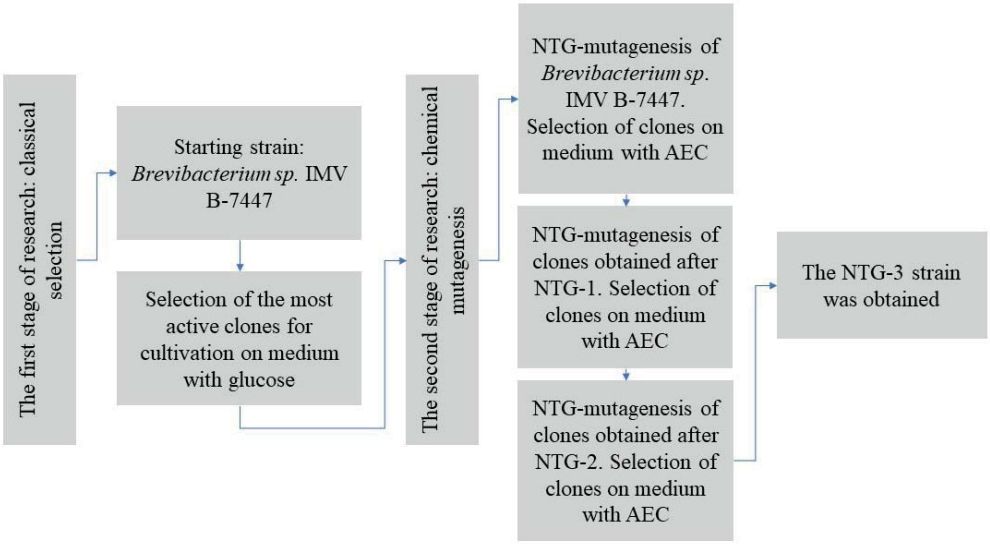

Fig. 3. **Scheme for the producing the strain with increased lysine accumulation**

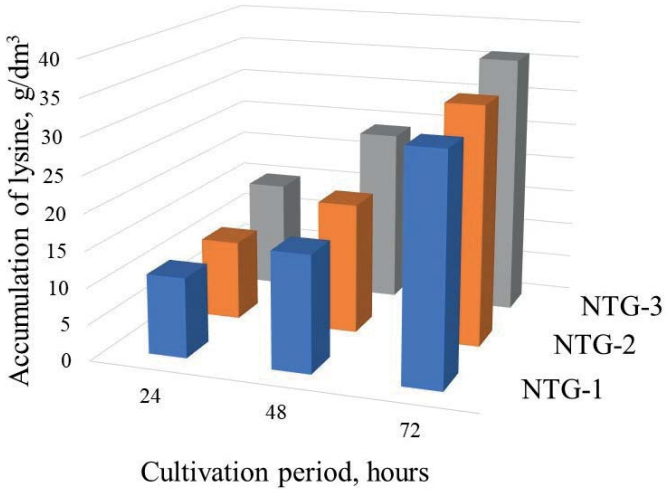

Fig. 4. **Dynamics of lysine accumulation by clones after chemical mutagenesis** ($P \leq 0{,}05$)

To develop the lysine technology and its further scaling, periodic cultivation with feeding was carried out in the Biostat B TWIN bioreactor.

During the cultivation of the strain, the accumulation of lysine in the culture medium was determined (Fig. 5).

On the first day of cultivation under periodic conditions, the producer strain used up to 25% of carbohydrates for biomass synthesis. Further, in spite of the background decrease in the growth rate, the cells actively synthesized lysine.

The rate of lysine accumulation by the producer strain *Brevibacterium* sp. IMV B-7796 was 0.96 g/dm^3/h. Within 69 hours, the strain accumulated 65±2 g/dm^3 lysine, and the bioconversion of carbohydrates was 48% . Further cultivation was not appropriate, the amount of lysine in the culture liquid decreased, which may be due to the use of lysine by the culture to accumulate biomass.

Conclusions

The effect of chemical mutagenesis (NTG) on cells of the *Brevibacterium* sp. IMV B-7797 strain was shown. Cell survival under the influence of NTG varied depending on its concentration and duration of action. Within two minutes of NTG exposure at a concentration higher than 200 µg/dm^3,

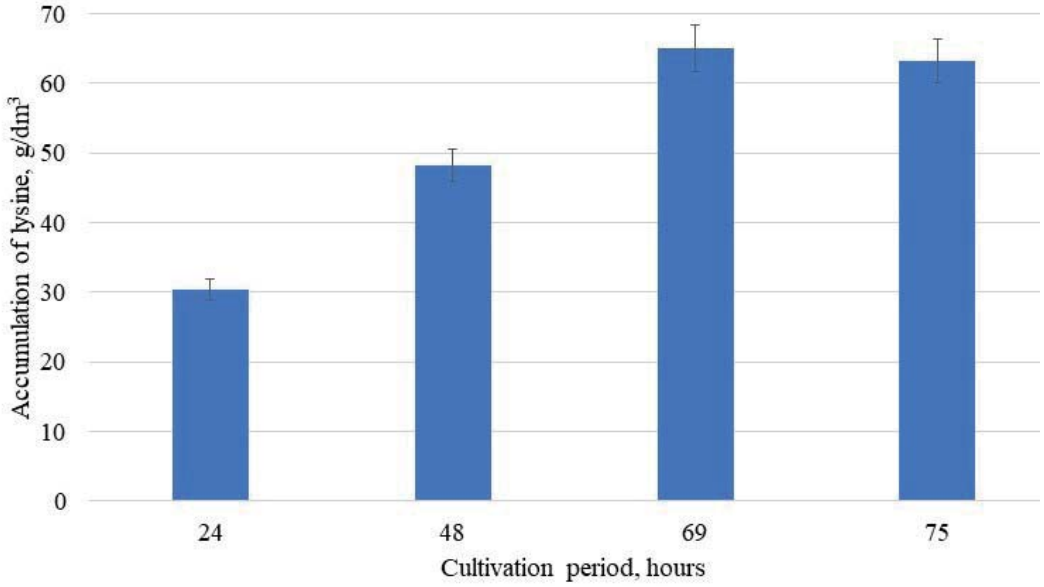

Fig. 5. **Accumulation of lysine by *Brevibacterium* sp. IMV B-7796 in a bioreactor**
Note: The initial concentration of lysine in the medium with the inoculum was 0.1 g/dm^3

no living cells remained. The greatest mutagenic effect was obtained using a NTG concentration of 100 μg/dm^3 and an exposure time of 20 minutes. In order to achieve the maximum accumulation of lysine, mutagenesis was carried out consecutively three times. A lysine-producing strain of *Brevibacterium* sp. IMV B-7796 with a lysine accumulation rate of 0.96 g/dm^3/h, which accumulated 65±2 g/dm^3 lysine within 69 hours, had a carbohydrate bioconversion of 48% under conditions of periodic cultivation with feeding. The strain *Brevibacterium* sp. IMV B-7796 is proposed to be used in the future to create a recombinant strain with increased accumulation of lysine, and to develop industrial lysine technology.

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This article contains no human or animal research conducted by any of the authors.

The authors declare no conflict of interest.

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ХІМІЧНИЙ МУТАГЕНЕЗ ШТАМУ-ПРОДУЦЕНТА ЛІЗИНУ *Brevibacterium sp.* ІМВ В-7447

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Мета роботи — отримати штам-продуцент із підвищеним накопиченням лізину за допомогою методу хімічного мутагенезу.

Методи. Для досягнення мети використовували метод оброблення хімічним мутагеном N-метил-N-нітро-N-нітрозогуанідином штаму-продуцента лізину з культивуванням отриманого клону та визначенням накопичення лізину в колбах і біореакторі.

Результати. Встановлено оптимальні концентрації та час дії мутагену для одержання мутантних мікроорганізмів і відібрано клони з максимальним накопиченням лізину. Мутагенез проводили послідовно тричі. Як результат, отримано ауксотрофний за лейцином та треоніном штам-продуцент лізину *Brevibacterium* sp. IMB B-7796 з максимальним накопиченням цільової амінокислоти.

Висновки. Отримано продуцент лізину *Brevibacterium* sp. IMB B-7796, що накопичував 65,0 г/дм^3 лізину в біореакторі за умов періодичного культивування з підживленням. Штам *Brevibacterium* sp. IMB B-7796 запропоновано для створення на його основі генетично-модифікованого штаму з підвищеним накопиченням лізину і подальшим використанням у промисловій технології лізину.

Ключові слова: *Brevibacterium* sp., лізин, хімічний мутагенез, штам-продуцент.

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THE INFLUENCE OF ACID PROTEIN PRECIPITANTS ON THE SPECIFICITY OF THE REACTION OF NINHYDRIN WITH AMINO ACIDS

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Aim. The purpose of the work was to determine the effect of trichloroacetic (TCA) and perchloric ($HClO_4$) acids on the result of ninhydrin reaction with various amino acids.

Methods. A standard method of amino acid detection using a ninhydrin reagent was applied. Optical spectra and density of reaction products were determined spectrophotometrically.

Results and conclusions. As a result, it was found that the studied acids change the spectral characteristics of the products of the ninhydrin reaction with amino acids. TCA significantly reduced the optical density of chromophores, and $HClO_4$ also led to a significant shift of the spectra of the reaction products into the short-wavelength region. An exception was the reaction with proline, as a result of which a well-defined maximum appeared in the product spectrum: $\lambda = 620$ nm in the presence of TCA and $\lambda = 515$ nm with $HClO_4$. At the same time, in the presence of $HClO_4$, the reaction became highly specific for proline.

The conditions for the ninhydrin reaction with proline upon addition of $HClO_4$ were analyzed in detail. As a result, a new method of highly specific determination of proline in the presence of other amino acids was proposed.

Key words: ninhydrin reaction, amino acids, proline, trichloroacetic acid, perchloric acid.

For practical purposes, proteinases are widely used in various industrial biotechnological processes [1–4] and medicine [5–7]. The reaction of amino acids with ninhydrin, which is a well-known reagent for proteins, peptides, and amino acids, is most often used to study proteolytic activity [8]. Moore and Stein made a significant contribution to the widespread use of the ninhydrin reaction [9]. These scientists analyzed in detail the main indicators and parameters of the reaction (properties of chromogenic products, duration of heat treatment and color preservation time, pH value, intensity of color and spectra of the products based on interaction with individual amino acids), proposed a method of color stabilization, etc. Thanks to the work of Moore and Stein, the method of determining amino acids using the ninhydrin reaction has become routine and is widely used in the chromatographic analysis of the amino acid composition of proteins, as well as in studying the activity of proteinases, collagenases, etc. [10].

Despite the ancient history of ninhydrin reaction, all its features have not yet been ascertained. Thus, we did not find detailed information in the literature about how protein precipitants — perchlorate ($HClO_4$) and trichloroacetic (TCA) acids — will affect the result of ninhydrin reaction with amino acids. These acids are usually used to separate the high-polymer molecules of substrate and enzyme from hydrolysis products [9], which is important for a more accurate analysis of proteolytic activity. Therewith, it should be emphasized that the ninhydrin reaction is not specific and the vast majority of amino acids form a chromogenic product with approximately the same optical density [8].

Thereby, the aim of the work was to determine how TCA and $HClO_4$ will affect the specificity and peculiarities of ninhydrin reaction with various amino acids (optical spectrum, duration of chromogenic products preservation, time of heat treatment, etc.).

Materials and Methods

L-amino acids (Serva), TCA and $HClO_4$, dimethylsulfoxide (DMSO), methyl cellosolve (MC), isopropanol of the "ag" or "rg"x qualification were used in the work. Ninhydrin was purified by double recrystallization from water.

The effect of TCA was studied at a final concentration of 4.5% and 10%, and the effect of perchloric acid was in the range of final concentration from 4.1% to 10.4%. It is known that the minimum indicated doses of these acids are sufficient for proteins separation and low-molecular products of their hydrolysis [11]. Therefore, lower concentrations of acids were not studied.

In the experiments, a scheme for detecting amino acids using a ninhydrin reagent was used as a standard, as described in [10]. As a control, a volume of water equivalent to the volume of acid precipitant used in the experiment was added to 0.5 ml of a 1 mM aqueous solution of amino acids. After 15–20 min ninhydrin solution was poured in and the mixture was incubated in a boiling water bath (95±3 °C). The volume of the ninhydrin reagent and the duration of heat treatment were varied depending on the experiment tasks. After cooling the mixture to room temperature (10 min), a solvent (ethanol, isopropanol, methylcellosolve, dimethylsulfoxide or a mixture of the last two) was added. The optical spectra and optical density of the reaction products were determined on a Cary 60 UV-Vis spectrophotometer in quartz cuvettes with an absorbing layer thickness of 1 cm.

The ninhydrin reaction result (chromogenic product yield) was expressed in units of optical density. Experiments were performed in 3–5 independent repetitions. Data processing was carried out using standard Microsoft Excel package: average arithmetic values and their standard errors were calculated, as well as graphs were constructed and approximation equations and their probability level were obtained.

Results and Discussion

It was found that the ninhydrin reaction result depended both on the applied precipitants and on the investigated amino acids. In the presence of TCA, for all amino acids (except imino acids), the optical absorption spectrum maximum of the reaction products remained unchanged and amounted to 570 nm, as in the standard method [10]. However, this acid in most cases significantly reduced the optical density (Table 1).

As it can be seen from the presented data (Table 1), it is difficult to find a connection between the structure of the amino acid and the effect of TCA on chromogenic products formation of the ninhydrin reaction. The formation of the colored product is significantly inhibited when studying amino acids of completely different groups (noted in Table 1 with a gray marker). Oxyproline in the studied conditions did not have a pronounced peak in the spectrum at all. At the same time, the minimum studied doses of TCA practically did not affect the analysis of glycine and alanine, and the effect of high acid concentration was relatively moderate.

Table 1. **The effect of TCA on the ninhydrin reaction result**

Reaction conditions	Researched amino acids *, **									
	Gly	Ala	Leu	Glu	Gln	Arg	Trp	His	Lis	Cys
Control, without TCA	1.50/ 100%	2.09/ 100%	2.10/ 100%	2.01/ 100%	1.73/ 100%	1.02/ 100%	1.20/ 100%	1.20/ 100%	1.55/ 100%	1.42/ 100%
4.5% TCA	1.48/ *99%*	2.00/ *96%*	0.41/ *20%*	0.42/ *20%*	1.16/ *66%*	0.20/ *20%*	0.16/ *20%*	0.73/ *60%*	0.66/ *43%*	1.25/ *88%*
10.0% TCA	1.20/ *80%*	1.44/ *67%*	0.00/ *0%*	0.00/ *0%*	0.16/ *9%*	0.00/ *0%*	0.00/ *0%*	0.08/ *7%*	0.00/ *0%*	0.24/ *17%*

Note: * — the value of the optical density in the maximum zone of the optical spectrum (λ = 570 nm) and ** — its level in the experiment compared to the control in % are indicated; experimental conditions — 1.5 cm^3 of ninhydrin reagent was added to 0.5 cm^3 of a 1 mM amino acid solution, the duration of heat treatment was 20 min, the final solvent of the reaction products: 3 cm^3 of a mixture of isopropanol and water in a 1:1 ratio.

A special picture was observed when determining proline. In the presence of TCA in the reaction mixture, a clearly defined wavelength maximum of $\lambda = 620$ nm appeard in the optical spectrum of the reaction products (Fig. 1). Furthermore, in the case of proline, an increase in TCA concentration led to an increase in the optical density of the resulting solution unlike the options with other amino acids.

Compared with TCA, the influence of $HClO_4$ on the ninhydrin reaction result was more significant (Fig. 2). For all investigated amino acids, the maximum characteristic of the reaction under standard conditions ($\lambda = 570$ nm) completely disappeared in the optical absorption spectra of the chromogenic reaction product. At the same time, the total optical density also decreased significantly. The reaction with lysine and tryptophan revealed an increase in optical density in a highly stretched region in the range of $\lambda = 460–500$ nm. Only with proline, the reaction product spectrum had a well-defined maximum at $\lambda = 515$ nm, absent for other amino acids, including oxyproline.

Thus, the addition both perchloric acid and TCA ensured the specificity of the reaction to proline.

The ninhydrin reagent used as a standard [10] had a pH value of 5.5. After adding TCA or $HClO_4$ to the amino acid solution, the acidity of the amino acid mixture with the ninhydrin reagent increased significantly and was ≤ 2. The peculiar behavior of proline in the ninhydrin reaction in an acidic medium was noted long ago [12]. Various methods of determining proline using an acidic ninhydrin reagent were proposed, of which the Bates' et al. one became the most popular [13].

Without special analytical studies, it is difficult to estimate the chemical mechanism of action concerning the investigated precipitating acids on the ninhydrin reaction result with amino acids. Perhaps, being strong oxidants and reagents capable of nucleophilic substitution, they affect the key stages of the reaction such as oxidation of amino acids and condensation of the resulting products. The role of $HClO_4$ as a cyclization catalyst is also known [14]. Friedman and Sigel [15] showed that the reaction of amino acids with ninhydrin depends both on the basicity of amino groups and steric features of amino components.

The high specificity of proline detection with a ninhydrin reagent in the presence of $HClO_4$ prompts detailed analysis and optimization of the main conditions for the procedure. First of all, it is necessary to analyze the following points:

1) to choose the optimal concentration of perchloric acid,

2) to specify the dependence of the reaction results on the duration of heat treatment with a ninhydrin reagent,

3) to determine the shelf life of the chromogenic product,

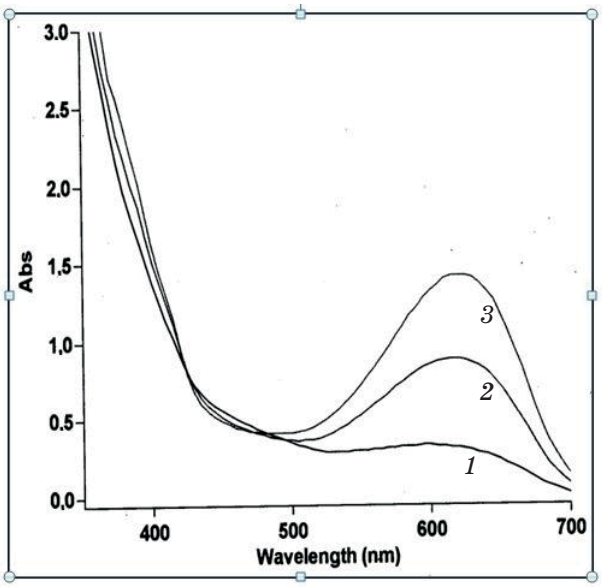

Fig. 1. **Spectrum of proline reaction products with ninhydrin in the presence of TCA:**
1 — proline without TCA, *2* — proline with 4.5 % TCA, *3* — proline with 10 % TCA, experimental conditions: 0.5 cm^3 of 1 mM imino acid solution, 1.5 cm^3 of ninhydrin solution, heat treatment duration was 20 min, after cooling 3 cm^3 of isopropanol mixture and water (1:1) were added.

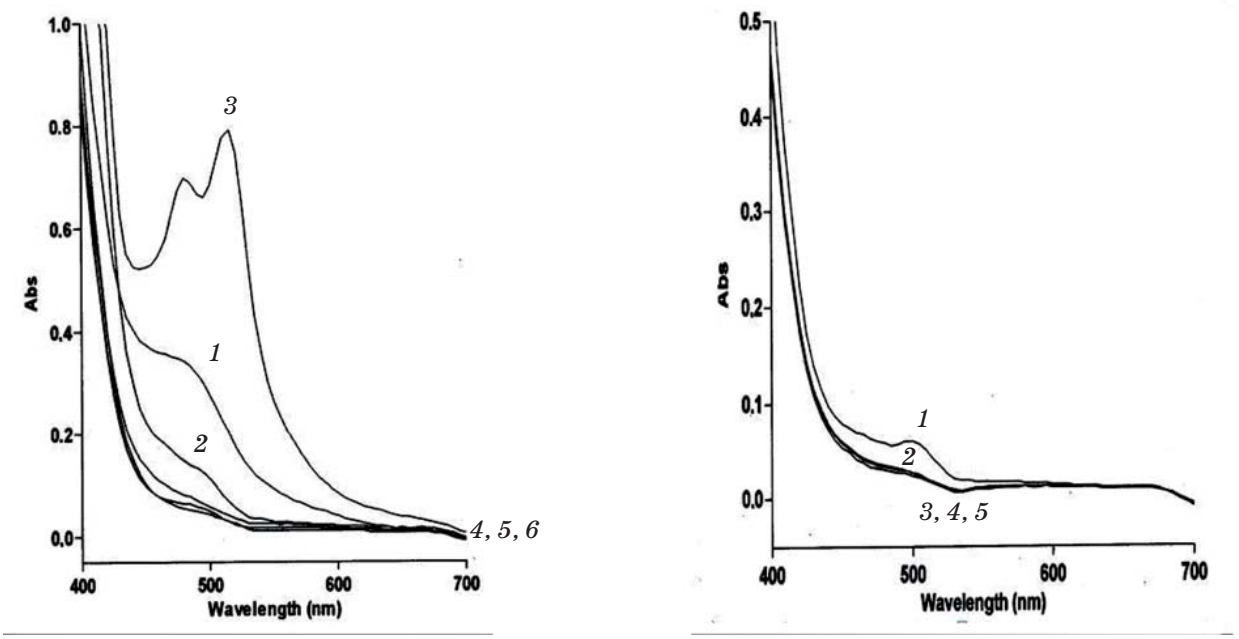

Fig. 2. **Reaction products spectra of the studied amino acids with ninhydrin in the presence of 4.1% $HClO_4$:**
a) *1* — lysine, *2* — tryptophan, *3* — proline, *4* — histidine, *5* — cysteine, *6* — glutamine;
b) *1* — oxyproline, *2* — arginine, *3* — glycine, *4* — alanine, *5* — leucine;
experimental conditions: 0.5 cm^3 of 1 mM amino acid solution, 1 ml of ninhydrin solution, heat treatment duration was 40 min, after cooling 2 cm^3 of DMSO and MC (1:1) mixture were added.

4) to find out the optimal ratio of the main components of the reaction mixture (non-hydric reagent and solvent),

5) to deafine the effect of other amino acids on the detection of proline (interference of proline with other components of proteins),

6) to find the linear dependence region of the optical density on proline concentration.

Elucidation of the above will contribute to a method development for proline specific determination. This method will primarily help to study the importance of this aminoacid in the protective reactions of organisms, and will also be useful for studying the functioning of proteinases, especially collagenases. The results of the search for the optimal concentration of $HClO_4$ are shown in Fig. 3. As it can be seen, increasing the acid dose is responsible for a negative effect on the detection of the colored product. Perhaps this is related to the increased destruction of the aminoacid by high concentrations of perchloric acid.

The influence of heat treatment duration on the final product optical density is given in Fig. 4, from which it can be seen that the reaction reaches a maximum in an hour. According to various protocols, the recommended processing time was from 20 to 60 min [16]. Thus, the obtained results fit into the specified terms.

The weak point of the ninhydrin reaction was the relative instability of the colored products. Different ways of increasing their stability are known: addition of complexing cations (Co^{2+}, Cu^{2+}, Ca^{2+}, etc.), use of non-aqueous solvents, pH value choice [8, 9, 17, 18].

The results of different solvents using after heat treatment were compared, as well as pH value changes (Table 2). It can be seen that ethanol and isopropanol, as the most frequently used solvents for ninhydrin reaction products [8, 9, 16–18] did not provide color stability under our conditions. Thus, when using isopropanol, a finely dispersed chromophore precipitate was noticed in the reaction mixture already after an hour, which caused a decrease in optical density indicators. It is known that one of the colored products of the ninhydrin reaction, hydrindanthin, dissolves very poorly in aqueous media and is unstable. Reducing the acidity of the environment by adding alkali (options No. 3 and 4) did not give a positive result. The most effective solvents were DMSO, MC and their mixture. With their use, the chromophores remained stable under these conditions for an hour. Perhaps this was primarily due to the high solubility of these substances. The introduction of reducing agents (dithiothreitol or ascorbic acid) did not protect the reaction products, but on the contrary, worsened the result.

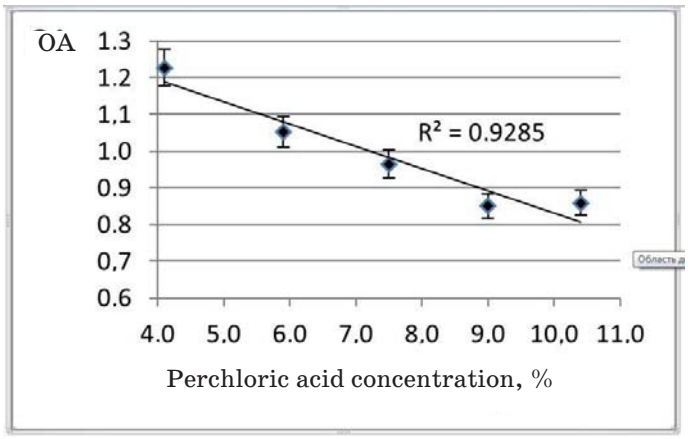

Fig. 3. **The influence of different concentrations of $HClO_4$ on the chromogenic product yield in the ninhydrin reaction**

Optical absorption (OA) at a wavelength of $\lambda = 515$ nm.
Experimental conditions: 0.050, 0.075, 0.100, 0.125, 0.150 cm^3 of 45% $HClO_4$, 1 cm^3 of ninhydrin reagent were added to 0.5 cm^3 of 1 mM proline solution; after heat treatment (45 min) and cooling (10 min), 2 cm^3 of a mixture of DMSO and MC (1:1) was added. R^2 is the reliability value of the linear approximation.

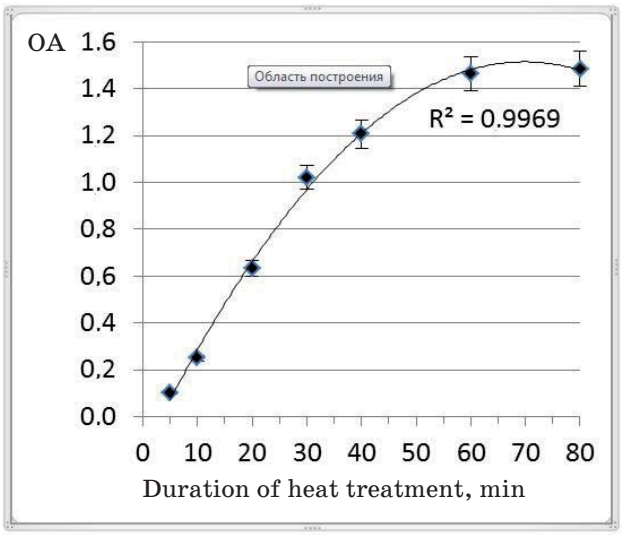

Fig. 4. **Quantity dependence of the obtained colored product (optical absorption, 515 nm) on the duration of treatment in a boiling water bath (min)**

Experimental conditions: 0.5 cm^3 of 1 mM proline solution with 0.05 cm^3 of 45% $HClO_4$, 1 cm^3 of ninhydrin reagent; after the end of heat treatment and cooling (10 min), 2 cm^3 of a mixture of DMSO and MC (1:1) was added. R^2 is the reliability value of the polynomial approximation.

It also was found that the stability of the ninhydrin reaction products depended not only on the solvents used, but on the duration of heat treatment as well (Table 3). As it can be seen from the presented data, stability was achieved when the reaction mixture was heated for 30 min and above. At the same time, high values of optical density, convenient for analysis, were provided.

The ratio of its components is important for the reaction optimization. Naturally, an increase in the relative dose of the ninhydrin reagent and a decrease in the amount of solvent (Tables 4, 5) leads to an increase in the value of the optical density of the reaction mixture. From the data presented, a tendency to stabilize the color could be observed with an increase in the relative proportion of the solvent. However, excessive dilution of the mixture with a solvent significantly impaired the analytical significance of the measurement.

From the given data (Tables 3–5), it can also be assumed that the ninhydrin reaction requires a certain time to stabilize the chromogenic products. This requires about 30 min after heat treatment. Thus

Table 2. **Stability dependence of ninhydrin products with proline on the solvents composition in perchloric acid presence**

No. of option	Reaction conditions: the solvent composition of the reaction products; the final pH value in the mixture	Optical density change (%) in time after the start of measurements (min)						
		0	30	60	90	120	150	180
1	Ethanol + water (2:1); pH ≤ 2	100*	87**	80**	«–»	«–»	«–»	«–»
2	Isopropanol+water (1:1); pH ≤ 2	100*	53**	36**	«–»	24**	«–»	«–»
3	Isopropanol+water (1:1); pH 5.5–6.5	100*	55**	39**	«–»	28**	«–»	«–»
4	Isopropanol+water (1:1)+9 мМ NaOH; pH 5.5–6.5	100*	49**	41**	«–»	29**	«–»	«–»
5	DMSO; pH ≤ 2	100*	93**	90**	87**	81**	77**	73**
6	MC; pH ≤ 2	100*	89**	80**	76**	67**	65**	63**
7	DMSO + MC (1:1); pH ≤ 2	100*	97**	90**	89**	67**	67**	62**
8	DMSO + 10 мМ dithiothreitol; pH ≤ *2*	100*	77**	70**	66**	63**	61**	57**
9	DMSO + 10 мМ ascorbic ac; pH ≤ 2	100*	54**	37**	29**	22**	20**	«–»

Note: * the initial measurement of the optical density of the reaction products was carried out after 30 min after completion of the reaction, the value of which was taken as 100 %;

** — the final value of the optical density in comparison with the initial value;

«–» — measurements were not performed.

Experimental conditions: 0.5 cm^3 of a 1 mM solution of proline with 0.05 cm^3 of 45% $HClO_4$ (in option №. 3, another 0.05 cm^3 of 0.1 N NaOH was added), 1 cm^3 of ninhydrin reagent; after heat treatment (30 min) and cooling (10 min), 2 cm^3 of a certain solvent was added (in option №. 4, 0.2 cm^3 of 0.1 N NaOH was added to the solvent).

Table 3. **Color preservation of the ninhydrin products with proline in the presence of perchloric acid at different times of heat treatment**

Duration of heat treatment (min)	Initial optical density	Optical density change (%) in time after completion of the reaction (min)				
		10	30	40	60	120
5	0.120± 0.004	100*	79**	74**	73**	70**
10	0.250± 0.008	100*	89**	87**	85**	79**
20	0.700± 0.022	100*	94**	93**	90**	83**
30	1.100± 0.034	100*	95**	94**	91**	83**
40	1.260± 0.038	100*	95**	94**	91**	83**
60	1.440± 0.044	100*	96**	93**	91**	83**
120	1.510± 0.045	100*	95**	93**	91**	84

Note: * — the initial measurement of the optical density of the products was carried out after 10 min after completion of the reaction, the value of which was taken as 100%;

** — the final value of the optical density in comparison with the initial one.

Experimental conditions: 0.5 cm^3 of 1 mM proline solution with 0.05 cm^3 of 45% $HClO_4$, 1 cm^3 of ninhydrin reagent; after the end of heat treatment and cooling (10 min), 2 cm^3 of DMSO+MC (1:1) mixture was added.

Table 4. **Products stability dependence of the ninhydrin reaction with proline in the presence of perchloric acid on the ratio of proline solution volumes and ninhydrin reagent volume and the volume ratio to the volume of reaction solvent mixture**

Ninhydrin reagent volume, cm^3	Volume ratio of the proline solution to the volume of the ninhydrin reagent	Solvent volume, cm^3	Solvent volume ratio to the total volume of the mixture	Initial optical density	Changes of optical density (%) over time, min	
					30	60
0.25	1.0 : 0.5	3.75	0.83 : 1.00	0.42± 0.01	100	107*
0.50	1.0 : 1.0	3.50	0.78 : 1.00	0.61± 0.02	100	102*
1.00	1.0 : 2.0	3.00	0.67 : 1.00	0.97± 0.02	100	98*
1.50	1.0 : 3.0	2.50	0.56 : 1.00	1.11± 0.04	100	96*
2.00	1.0 : 4.0	2.00	0.44 : 1.00	1.38± 0.04	100	93*

Note: the initial measurement was made after 30 min after completion of the reaction and cooling (10 min), which value was taken as 100%;

* — optical density value compared to the initial one.

Experimental conditions: 0.5 cm^3 of 1 mM proline solution + 0.05 cm^3 of 45% $HClO_4$; the volume of the reaction mixture in all versions is constant (4.50 cm^3); duration of heat treatment — 45 min; solvent was a mixture of DMSO+MC (1:1).

Table 5. **Ninhydrin reaction optimization with proline in the presence of perchloric acid according to the solvent volume ratio to the reaction mixture volume**

Solvent volume, cm^3, (total volume of the reaction mixture, cm^3)	Solvent volume ratio to the volume of the reaction mixture	Initial optical density	Changes of optical density value (%) of the reaction products in relation to the initial measurement during observation (min)		
			30 min	60 min	90 min
1.5 (3.05)	1:1.0	1.09± 0.04	100	98*	94*
2.0 (3.55)	1:1.3	1.04± 0.03	100	98*	93*
2.5 (4.05)	1:1.7	1.00± 0.03	100	99*	95*
3.0 (4.55)	1:2.0	0.98± 0.03	100	99*	97*
4.0 (5.55)	1:2.7	0.83± 0.03	100	102*	100*
4.5 (6.05)	1:3.0	0.83± 0.03	100	105*	105*

Note: the initial measurement was made after 30 min after completion of the reaction and cooling (10 min), the value of which was taken as 100%; * — optical density value compared to the initial one. Experimental conditions: 0.5 cm^3 of 1 mM proline solution + 0.05 cm^3 of 45% $HClO_4$; volume of ninhydrin reagent — 1 cm^3; the total volume of the reaction mixture in different versions is not the same; duration of heat treatment was 45 min; the solvent was a mixture of DMSO+MC (1:1).

(Tables 4, 5), it is exactly after this time the optical density of products slowly decreases (no more than 1–3% per hour.

Based on the experiments, we can recommend the most optimal conditions for conducting the ninhydrin reaction with proline in the presence of perchloric acid: to 0.5 cm^3 of the proline solution it need to add $HClO_4$ solution to a final concentration of 4–4.1%, to add 1 cm^3 of the ninhydrin reagent (according to the prescription work 10), to hold in a boiling water bath for up to 60 min, to cool to room temperature and at the end to add 3 cm^3 of the DMSO + MC mixture. To make the measurement at a wavelength of 515 nm 30 min later.

The proposed method is also convenient from the point of view that the same ninhydrin reagent can be used both for the specific determination of proline and for the determination of other amino acids by conventional methods.

Despite the fact that separately all the studied amino acids did not show a noticeable reaction with ninhydrin in the presence of perchloric acid, it is necessary to check their

effect on the detection of proline by analyzing mixtures of amino acids (Table 6). To assess the interference of amino acids with proline, the deviation degree of the reference values obtained for proline from the optical density indicators of the mixtures was calculated. As can be seen from the table, a significant part of the investigated amino acids in equimolar ratios with proline (glycine, alanine, oxyproline, arginine, glutamic and aspartic acids, etc.) have a weak influence on the determination of proline.

The SH group is a nucleophile, therefore it can participate in nucleophilic addition or substitution reactions and compete with NH_2 groups of amino acids for the reaction with ninhydrin [15, 17]. As a result of such competition, the amount of chromogen in the ninhydrin reaction of proline in the presence of methionine and cysteine was decreased. The increase in color with lysine is associated with the interaction of ninhydrin with the ε-amino group of the amino acid [8].

For comparison, a similar analysis was performed according to the widely used method of Bates et al. [13], which is considered a reference. In the most cases, the proposed method provided greater specificity for proline determination in the presence of other amino acids and was superior to the reference method.

A number of successive dilutions of proline were prepared to construct a calibration graph. Proline solutions with a concentration from 0.01 mmol/dm^3 (0.576 μg in the analyzed sample of 0.5 cm^3) to 20.00 mmol/dm^3 (1151.300 μg in the analyzed sample) were analyzed.

The range of application of the proposed method, that is, the preservation of the linear relationship between the optical density and the amount of amino acid,

Table 6. **Proline interference with other amino acids in reaction with ninhydrin in the presence of perchloric acid**

Amino acids	The degree of deviation of the optical density (%) of the mixture of amino acids in relation to the values for proline	
	The original method	The method of Bates et al., 1973
Proline 1 мМ	0.0	0.0
Proline 1 мМ + glycine 1 мМ	+0.5	+7.2
Proline 1 мМ + alanine 1 мМ	+1.6	+18.2
Proline 1 мМ + valine 1 мМ	−4.8	+14.5
Proline 1 мМ + oxyproline 1 мМ	+0.2	+3.3
Proline 1 мМ + threonine 1 мМ	−5.0	+5.8
Proline 1 мМ + methionine 1 мМ	−8.3	+19.6
Proline 1 мМ + arginine 1 мМ	−1.2	−4.5
Proline 1 мМ + asparagine 1 мМ	−1.6	+11.9
Proline 1 мМ + glutamine 1 мМ	−1.8	−8.6
Proline 1 мМ + lysine 1 мМ	+5.8	+4.9
Proline 1 мМ + leucine 1 мМ	−2.0	+3.2
Proline 1 мМ + isoleucine 1 мМ	+0.2	+9.4
Proline 1 мМ + cysteine 1 мМ	−3.3	−3.2
Proline 1 мМ + histidine 1 мМ	−6.0	+19.7
Proline 1 мМ + phenylalanine 1 мМ	−5.9	−1.2
Proline 1 мМ + tryptophan 1 мМ	+1.6	−18.6
Proline 1 мМ + tyrosine 1 мМ	+1.5	+8.3
Proline 1 мМ + glutamic acid 1 мМ	−1.2	+5.3
Proline 1 мМ + aspartic acid 1 мМ	+1.3	−12.9

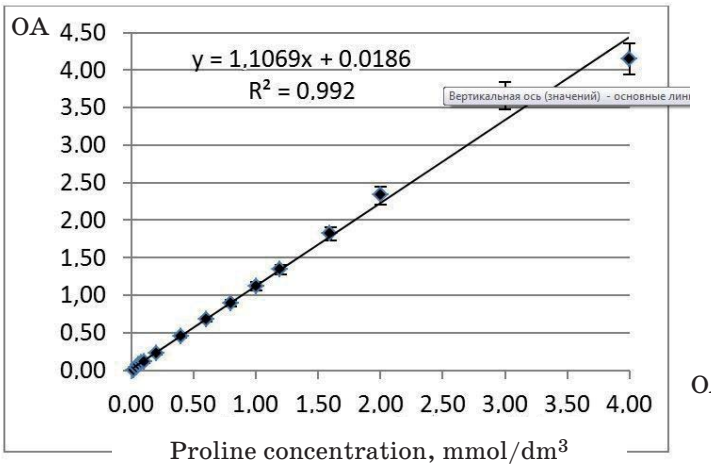

Fig. 5. **Calibration graph for proline:** OA — optical density; the specified approximation equations and its reliability level (R^2).

is maintained up to a concentration of 4.00 mmol/dm^3 (230.26 μg in the analyzed sample) with an approximation coefficient of $R^2 = 0.992$ (Fig. 5). Proline detection limit: 0.01 mmol/dm^3 (0.576 μg in the analyzed sample); the limit of quantitative determination is 0.02 mmol/dm^3 (1.151 μg in the analyzed sample). The average standard error (SE) for the entire range of measurements is 3.32% of the optical density of the samples.

Thus, the study of the effect of acid precipitants of proteins made it possible to propose a method for the specific determination of proline using a standard ninhydrin reagent. The main results of the work can be formulated as follows.

Trichloroacetic (TCA) and perchloric ($HClO_4$) acids change the spectral characteristics of the products of the ninhydrin reaction with amino acids.

In the presence of TCA, the optical density of reaction products decreases for most amino acids without TCA shifting the maximum of the optical spectrum. In the presence of $HClO_4$, for most amino acids, the maximum characteristic of the ninhydrin reaction under standard conditions ($\lambda = 570$ nm) completely disappears and the optical spectrum of the chromophore shifts to the short-wavelength zone. At the same time, the total optical density of the chromophore also decreases significantly.

The exception is the reaction with proline, as a result of which a well-defined maximum appears in the product spectrum: $\lambda = 620$ nm in the presence of TCA and $\lambda = 515$ nm with $HClO_4$. Moreover, in the presence of perchloric acid, the reaction becomes highly specific for proline.

On the basis of the obtained results, a method of highly specific determination of proline was developed [19].

In our opinion, the use of the proposed specific method for the determination of proline in combination with ways specific to other amino acids (oxyproline and glycine) and in combination with standard methods for the determination of amino acids can contribute to the elucidation of the features of recognition sites in collagen molecules during their hydrolysis by collagenases. This approach can also be useful for studying the mechanism of action of other proteinases.

This work was carried out in the framework of the theme of Bogatsky Physico-chemical Institute of the National Academy of Science of Ukraine "Research of enantioselective hydrolysis enzyme systems and tyrosinase inhibitors for the creation of new biologically active compounds" (02.01.2020–31.12.2022).

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ВПЛИВ КИСЛОТНИХ ОСАДЖУВАЧІВ ПРОТЕЇНІВ НА СПЕЦИФІЧНІСТЬ РЕАКЦІЇ НІНГІДРИНУ З АМІНОКИСЛОТАМИ

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Мета. Визначити вплив трихлороцтової (ТСА) та перхлоратної ($HClO_4$) кислот на результат реакції нінгідрину з різними амінокислотами.

Методи. Застосовували стандартний метод виявлення амінокислот з використанням нінгідринного реагента. Оптичні спектри та густину продуктів реакції визначали спектрофотометрично.

Результати та висновки. Виявлено, що досліджувані кислоти змінюють спектральні характеристики продуктів нінгідринної реакції з амінокислотами. ТСА суттєво знижує оптичну густину хромофорів, а $HClO_4$ до того ж призводить до значного зміщення спектрів продуктів реакції у короткохвильову область. Винятком є реакція з проліном, у результаті якої у спектрі продуктів з'являється чітко виражений максимум: $\lambda = 620$ нм за присутності ТСА та $\lambda = 515$ нм з $HClO_4$. При цьому за присутності $HClO_4$ реакція стає високоспецифічною щодо проліну.

Аналізовано умови проведення нінгідринної реакції з проліном при додаванні $HClO_4$. Запропоновано спосіб високоспецифічного визначення проліну за присутності інших амінокислот.

Ключові слова: нінгідринна реакція, амінокислоти, пролін, трихлороцтова кислота, перхлоратна кислота.

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OPTIMIZATION OF THE EVALUATION METHOD OF THE PERFORMANCE OF THERAPY USING INDIRECT ACTION ANTICOAGULANTS

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Aim. Treatment by indirect anticoagulants (vitamin K antagonists) requires a personalized approach for controlling the overall level of prothrombin and the accumulation of its decarboxylated forms. The purpose of this work was to optimize the method for monitoring of the therapy with indirect anticoagulants.

Methods. An analysis was performed of 41 blood plasma samples from patients with cardiovascular pathologies. Activated partial thromboplastin time (APTT), prothrombin time, ecamulin time, statistical data analysis ("Statistica 7") have been used.

Results. APTT test allowed identifying the individual sensitivity of patients to indirect anticoagulants. In particular, 20% of patients showed a decrease in the total level of prothrombin, which, together with the accumulation of decarboxylated forms, leads to a risk of bleeding. Individual insensitivity to the action of vitamin K antagonists was determined in 11% of patients.

Conclusion. To control the efficacy of indirect anticoagulants therapy, we developed test with ecamulin (protease from the venom of *Echis multisquamatis*) was used as a prothrombin activator, which can activate not only functionally active prothrombin, but also its decarboxylated forms. Use of ecamulin simultaneously with thromboplastin allows determining in the blood plasma the content of not only functionally active prothrombin, but also the total level of prothrombin, which makes it possible to control the accumulation of decarboxylated prothrombin.

Key words: prothrombin, vitamin K, indirect anticoagulants, thrombolytic therapy.

Currently, four classes of antithrombotic preparations are used clinically for the prevention and treatment of thrombosis: direct anticoagulants (heparin, low molecular weight heparins, inhibitors of thrombin and factor Xa); indirect anticoagulants (inhibitors that affect vitamin K-dependent clotting factors); antiplatelet agents (including non-steroidal anti-inflammatory drugs and clopidogrel that affect platelet adhesion and aggregation); thrombolytic drugs (agents that activate the fibrinolytic system, through the conversion of plasminogen to plasmin) [1–3].

A special place in the prevention of thrombosis belongs to oral anticoagulants, or indirect anticoagulants (IA), which are antagonists of vitamin K. The latter is necessary for the synthesis of functionally active coagulation factors, namely the vitamin K-dependent proteins: prothrombin, factors VII, IX, X, proteins C and S. These factors are key components of the coagulation cascade, and their functional activity determines the hemostatic potential of the blood (Fig. 1). As a result, of impaired post-translational γ-carboxylation of vitamin K-dependent

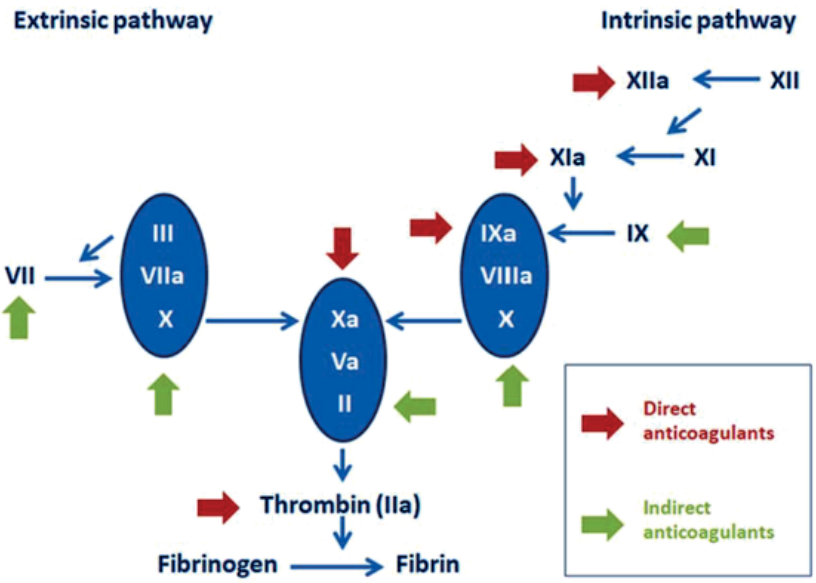

Fig. 1. **Actions of anticoagulants on the factors of the blood coagulation system [8]**
Indirect anticoagulants disrupt the carboxylation of blood coagulation factors, thereby preventing the activation of proenzymes (prothrombin, factor X, factors VII and IX). In contrast, direct anticoagulants act on the active enzymes of the coagulation cascade, predominantly thrombin or factor Xa.

proteins, a number of factors of the blood coagulation system enter the blood in the decarboxylated functionally inactive forms [4, 5]. That leads to a decrease in the procoagulant potential of the blood coagulation system, thereby contributing to the risk of bleeding. This requires constant and systematic determination of the content/activity of vitamin K-dependent factors of the hemostasis system and determines the need for regular monitoring of the degree of hypocoagulation [1, 5–7].

Materials and Methods

APTT-reagent, thromboplastin (thromborel S, Siemens, Germany), $CaCl_2$ solution were purchased from Berichrom. Ecamulin was purified from the venom of *Echis multisquamatis* according to the method of Solovjev et al [8].

Collection of blood plasma of patients. Samples were taken from 41 patients with cardiovascular pathologies (aged 34–80, $n = 41$), who were given warfarin as anticoagulant therapy. Blood collection was performed during anticoagulant therapy. Platelet-poor blood plasma was prepared from citrated blood by centrifugation at 1200 g during 30 min. Sodium Citrate (3.8%) added immediately after collection to the whole blood at 1:9 ratio was used as an anticoagulant [9]. All work was done in accordance with the Declaration of Helsinki. Studies were conducted according to the Ethical Committee Approval No. 8 form 11.05.2018 (Shupyk National Medical Academy of Postgraduate Education of Ministry of Health of Ukraine).

Activated partial thromboplastin time. Activated partial thromboplastin time (APTT) was performed according to the following procedure: 0.1 ml of studied blood plasma was mixed with equal volume of APTT-reagent and incubated during 3 minutes at 37 °C. Then the coagulation was initiated by adding of 0.1 ml of 0.025 M solution of $CaCl_2$ and clotting time was monitored. Time of clotting was evaluated using coagulometer CT2410 (Solar, Belarus).

When clotting time in APTT-test was prolonged we performed the APTT mixing study (inhibitory correction probe) as follows: 0.05 ml of studied blood plasma was mixed with 0.05 ml of control blood plasma sample, 0.1 ml of APTT-reagent and incubated during 3 minutes at 37 °C. Blood clotting was detected as described above. Normalization of blood clotting time indicated the deficiency of the clotting factors, otherwise the accumulation of blood clotting inhibitors was assumed [10].

Mixing study determines if the patient has a factor deficiency or the presence of a factor-inhibiting antibody. Data are interpreted as Dr Castellone indicated in her work: if the plasma of the patient is suspected of being factor deficient, adding the pooled normal plasma will add back the deficient clotting factor,

and the APTT will correct itself. If there is no correction in the corresponding APTT, the plasma of the patient contains an inhibitor, which prevents the ability of the pooled normal plasma to correct itself [11].

Prothrombin time. Thromboplastin reagent (INR = 1.1) was measured as follows: clotting was initiated by mixing 0.1 ml of blood plasma with 0.1 ml of 0.025 M $CaCl_2$ and 0.1 ml of thromboplastin reagent, time of clotting was monitored. Thromboplastin acts through tissue factor pathway of coagulation and activates only carboxylated and uncleaved forms of prothrombin. Time of clotting was evaluated using coagulometer CT2410 (Solar, Belarus). Results of prothrombin test were presented as International normalized ratio (INR) calculated by formula: $INR = (Ap/An)^{isi}$, Ap — studied blood plasma clotting time; An — blood plasma clotting time of healthy control; ISI — international sensitivity index [9].

Ecamulin time. Ecamulin test is based on the application of ecamulin, prothrombin activator from the venom of *Echis multisquamatis*. Ecamulin activates prothrombin, des-gamma-carboxy-prothrombin and prethrombin 1 thus permitting the determination of total prothrombin level [12].

Results of ecamulin test were presented as ecamulin ratio (ER) calculated by formula: ER = Ap/An; Ap — studied blood plasma clotting time; An — blood plasma clotting time of healthy control.

Statistical data analysis. Statistical data analysis was performed using Microsoft Excel. All assays were performed in series of three replicates and the data were fitted with standard errors using "Statistica 7".

Results and Discussion

The state of the blood coagulation system was analyzed in patients ($n = 41$) with cardiovascular pathologies who underwent a course of anticoagulant therapy with IA (warfarin). The state of the blood coagulation system was monitored using the activated partial thromboplastin time (APTT) test. The clotting time of the blood plasma of these patients in the APTT test was increased by 1.5–2.5 times compared with the norm (45 s). APTT mixing test led to the normalization of clotting time, which indicated the accumulation of decarboxylated forms (PIVKA-proteins) of coagulation factors in the blood plasma of patients. To determine the content of functionally active prothrombin, which is a key component of the coagulation cascade, we used the prothrombin time (PT) diagnostic test with thromboplastin as a prothrombin activator. To assess the decarboxylated forms of prothrombin, we used the "ecamulin time" test, a PT test optimized by us, in which ecamulin (analogous to ecarin) was used instead of thromboplastin as an activator of prothrombin. The content of functionally active prothrombin was expressed as a prothrombin ratio (PR); the content of total prothrombin (functionally active and inactive) was expressed as an ecamulin ratio (EO).

Based on the data obtained, the patients were divided into three groups: group 1 — patients with an effectively selected dose of IA (the potential of the coagulation system is reduced by 35–55%), group 2 — patients who are tolerant to IA (the procoagulant potential is fully preserved), group 3 — patients with hypersensitivity to IA (overdose of IA preparations) (Fig. 2).

For patients of the first group, a decrease in PR by 35–55% (0.6) compared with the norm (1.0 ± 0.1) is seen, which indicates the accumulation of decarboxylated prothrombin and a decrease in blood coagulation potential.

In the blood plasma of the second group of patients (Fig. 2), decarboxylated prothrombin, the presence of which is due to vitamin K deficiency, was not detected (ER = PR), which indicates the tolerance to IA.

In patients of the third group, a decrease in functionally active prothrombin to 20–30% was revealed against the background of a low content of total prothrombin (less than 70%). Such a significant decrease in coagulation potential may be accompanied by bleeding, and in such patients the use of IA is contraindicated.

Recent studies show high efficiency of IAs in the prevention and treatment of thrombotic complications and in reducing the risk of thrombosis [13, 14]. However, the individual sensitivity of the patient necessitates selecting the dose of IA preparations and monitoring their performance, which is due both to the characteristics of the patient's condition (age, platelet function, concomitant diseases, nutrition, hypertension, stroke consequences, alcohol dependence), and the problem of compatibility of the drugs used [2, 3, 5].

It should be noted that the total level of prothrombin in the blood plasma of patients can vary between 90–110%. The decrease in the potential of the coagulation system (accumulation of decarboxylated prothrombin forms) must be determined taking into account the total level of prothrombin in the patient in question, and not the average level

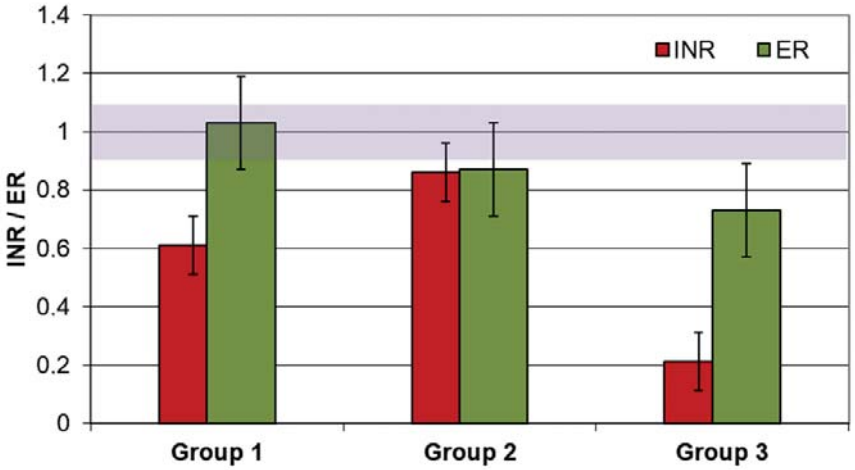

Fig. 2. **Prothrombin (PR) and ecamulin (ER) ratios, obtained from the analysis of blood plasma of patients who underwent IA therapy (peak of the treatment)**
Control (n = 12): INR and ER values are 1.0 ± 0.1

of prothrombin in the blood plasma of donors. The therapeutic interval for the content of functionally active prothrombin in the treatment of IA should be within 30–50%, which corresponds to an INR value of 2–3 [9].

The main control method throughout the clinical use of the IA has been and remains the determination of the prothrombin clotting time of blood plasma (in some cases, of the whole blood). The principle of the method is to determine the clotting time of blood plasma after the addition of thromboplastin in the presence of calcium ions. The test implements a number of successive and interrelated reactions, and the clotting time of blood plasma depends not only on the total rate of the process of activation of coagulation factors, but also on the presence of inhibitors of fibrin polymerization and thrombin inhibitors. Hence, with the obvious simplicity of the test itself, the evaluation of its results is a serious problem that has not been finally resolved to date. In addition, the determination of prothrombin time does not provide information on the presence and content of functionally inactive (decarboxylated) forms of prothrombin, since thromboplastin does not activate them. Therefore, to control the effectiveness of IA preparations, we developed test conditions in which ecamulin (a prothrombin activator from the venom of Echis multisquamatis) was used as a prothrombin activator. Ecamulin, unlike thromboplastin, is able to activate not only functionally active prothrombin, but also its decarboxylated forms [15–17], so the use of this method, which we have optimized, makes it possible to control the total level of prothrombin in blood plasma. Thus, with the parallel use of these two activators, it is possible to determine the content in the blood plasma of not only functionally active prothrombin, but also its decarboxylated forms.

The information content of our optimized method for monitoring the performance of IA therapy is evidenced by the results, on the basis of which the first group of patients was identified. According to the "ecamulin time" test, the content of functionally inactive forms of prothrombin is 40% of the total prothrombin level (Fig. 2). The content of functionally active prothrombin with effective therapy is reduced by 40–50% [15]. In addition, there is a decrease in the procoagulant potential, which indicates a correctly selected dose of the IA and high efficiency of treatment. The accumulation of functionally inactive forms of prothrombin in the blood plasma of patients of the first group also confirms a significant increase in the clotting time of blood plasma in the APTT screening test.

Comparative analysis of the results obtained during the activation of prothrombin in the blood plasma of patients with thromboplastin and ecamulin allowed us to identify a group of patients with low sensitivity to IA drugs (group 2). This conclusion was made on the basis that the level of prothrombin determined using thromboplastin and ecamulin was the same, which indicates the absence of decarboxylated forms of prothrombin. In such patients, either the dose of IAs should be increased to control the occurrence of decarboxylated prothrombin, or the IAs should be discontinued in favor of other anticoagulants.

For patients of the third group, there is a risk of bleeding: the content of total prothrombin is <70%, and the level of functionally active prothrombin is reduced to 20–30%. The reason for the decrease in the level of the latter may be the high sensitivity to the IAs. In these patients, IAs are contraindicated [18]. Perhaps, in such cases, the dose of IAs should be reduced and additional monitoring is needed to determine the reasons for the decrease in the total level of prothrombin.

It should also be noted that different sensitivity of patients to IAs, in particular to warfarin, can be genetically determined. Therefore, an individual approach to the dosage of IA based on the results of genetic testing can contribute to reducing the risk of hemorrhagic complications [13, 19].

Although the determination of prothrombin time is a generally accepted method for monitoring the IA effects, it alone is not enough. From a practical point of view, we consider it necessary to simultaneously perform the "ecamulin time" test, which allows us to determine the presence of functionally inactive (decarboxylated) forms of prothrombin. The need to control the content of decarboxylated forms of prothrombin is due to the individual sensitivity of patients to IAs.

Using an individualized test-based approach to IA dosage may help reduce the risk of hemorrhagic complications.

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ОПТИМІЗАЦІЯ МЕТОДУ ОЦІНЮВАННЯ ЕФЕКТИВНОСТІ ТЕРАПІЇ З ВИКОРИСТАННЯМ АНТИКОАГУЛЯНТІВ НЕПРЯМОЇ ДІЇ

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Лікування непрямими антикоагулянтами (антагоністами вітаміну К) потребує індивідуального підходу для контролю загального рівня протромбіну та накопичення його декарбоксильованих форм.

Мета. Оптимізувати метод моніторингу терапії непрямими антикоагулянтами.

Методи. Проведено аналіз 41 зразка плазми крові пацієнтів із серцево-судинною патологією. Використано методи лабораторної діагностики для визначення активованого часткового тромбопластинового часу (АЧТЧ), протромбінового часу (ПЧ), екамулінового часу (ЕЧ), статистичний аналіз даних («Statistica 7»).

Результати. Тест АЧТЧ дав змогу виявити індивідуальну чутливість пацієнтів до непрямих антикоагулянтів. Зокрема, у 20% пацієнтів виявлено зниження загального рівня протромбіну, що разом з накопиченням його декарбоксильованих форм призводить до розвитку кровотечі. Індивідуальну нечутливість до дії антагоністів вітаміну К було визначено в 11% пацієнтів.

Висновок. Для контролю ефективності терапії непрямими антикоагулянтами нами розроблено тест, в якому як активатор протромбіну використано екамулін (протеаза з отрути *Echis multisquamatis*), який може активувати не тільки функціонально активний протромбін, але і його декарбоксильовані форми. Застосування екамуліну одночасно з тромбопластином дає змогу визначати в плазмі крові вміст не тільки функціонально активного протромбіну, але й загального рівня протромбіну, що уможливлює контроль накопичення декарбоксильованого протромбіну.

Ключові слова: протромбін, вітамін К, непрямі антикоагулянти, тромболітична терапія.