

UDK 54.057:547.567:615.273

N. Raksha, PhD, D. Gladun, PhD stud.
Taras Shevchenko National University of Kyiv, Kyiv, Ukraine

DETERGENT-STABLE PROTEASES FROM THE ANTARCTIC SCALLOP *ADAMUSSIUM COLBECKI*

Detergent-stable proteases were found in crude extract of the Antarctic scallop Adamussium colbecki. The results of gelatin zymography indicated the availability of the proteases which able to act in the presence of SDS, whereas plasmin and miniplasmin maintained their activity toward gelatin only after complete removing of detergent. Total proteolytic activity, represented by casein hydrolysis was within the control level after incubation of crude extract with detergents such as Tween 80 and SDS whereas trypsin activity was significantly reduced.

Keywords: marine hydrobiont *Adamussium colbecki*, SDS, Tween 80, ethanol, total proteolytic activity.

Introduction. Proteases are an unique class of enzymes, since they have immense physiological as well as commercial importance. They possess both degradative and synthetic properties. Enzyme-catalyzed reactions play important roles in various industrial processes and proteases represent one of the three largest groups of industrial enzymes. They have extensive applications in a range of industrial and household products including detergents, food, leather, silk, pharmaceuticals industries and bioremediation processes [13, 7]. Proteases are also useful and important components of biopharmaceutical products such as enzyme contact-lens cleaners and enzymatic debriders [6, 2]. Proteolytic enzymes also offer a gentle and selective debridement, supporting the natural healing process in the successful local management of skin ulcerations by the efficient removal of the necrotic material. The demand for industrial enzymes progressively increased during the last decades. It includes the quantity of enzyme protein as well as the quality and purity of enzyme preparations.

Since proteases are physiologically necessary, they occur ubiquitously in animals, plants, and microbes. The enzymes can be obtained from various sources and the nature of the source provides the availability, the cost, the ease of recovery and other factors. For this reason many investigators have focused their attention on marine organisms, which have a significant potential of delivering valuable enzymes. It should be noted that in response to environmental factors like salinity of ocean waters, low light or its absence, high pressure, low temperature marine organisms and particularly hydrobionts from the Antarctic region have developed highly specialized biochemical adaptations. It provides not only considerable structural and functional diversity of marine hydrobionts biologically active compounds but also the presence of enzymes with unique structures and activities. So, proteases from cold-water organisms possess greater proteolytic activity toward native protein substrates, lower activation energy for catalysis and often have higher catalytic activity at very low temperatures, e.g. 4°C, compared with proteases from warm-blooded animals [11, 5]. Despite indisputable practical interest, cold-active enzymes also represent a valuable model for fundamental researches of protein folding and catalysis.

Many industrial applications of proteases require enzymes with properties that are non physiological. For example enzymes stable in organic solvents and/or detergents are of special interest for industrial applications [12]. Reports on a few enzymes that are naturally stable and also exhibit high activities in the presence of organic solvent have emerged recently [3].

In recent years, special attention has been paid to hydrobionts from the Antarctic region which are poorly explored and potentially could be a valuable source of new bioactive compounds. We have chosen for our study the Antarctic scallop *Adamussium colbecki*. This hydrobiont is one of the most common bivalves in Antarctica which is widely spread not only along the coast, but also can be

found at depths from 3 to nearly 1500 meters down into the ocean [14]. In our work we examined *A. colbecki* for the presence proteases stable in detergents and solvents.

Materials and methods. The specimens of *A. colbecki* (n = 35) were collected near the island Galindez (geographical coordinates – 65°15' south latitude, 64°15' west longitude) of Argentine Islands archipelago. The materials were collected by the XVII, XVIII Ukrainian Antarctic expeditions on March 2012 – April 2013, March 2013 – April 2014, respectively. After collection the scallops were immediately frozen in liquid nitrogen to prevent enzyme deterioration and stored at -80°C. The samples were brought to the laboratory frozen. The mass of hydrobiont was measured and the samples were homogenized with sequential addition of liquid nitrogen and extraction buffer – 0.1 M Na-phosphate buffer containing 0.15 M NaCl, 0.15 mM EDTA, 2 mM PMSF and 0.1% Triton X-100, pH 7.4. Samples were homogenized using blender (Braun, Germany) for 5 min and then centrifuged (Allegra 64 R Centrifuge, Beckman Coulter, USA) at 10 000 g for 60 min at 4°C. The resulting supernatant either used immediately or lyophilized. Thus obtained primary material can be stored for a long time at a temperature of -20°C without losing the functional properties of proteins and peptides.

Protein quantitation. Protein concentration was determined according to the method of Bradford [1], using bovine serum albumin as the standard protein and measuring the absorbance at 595 nm.

Zymography assay. Zymography was carried out according to the method [10]. The separating gel solution (12 %, w/v) was polymerized in the presence of gelatin (1 mg/ml). Samples for zymography were dissolved in the standard SDS-PAGE buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% (v/v) glycerol, and 0.002% bromophenol blue) under non-reducing conditions (no boiling and no reducing agents). After electrophoresis, the gels were soaked in 2.5% Triton X-100 solution with shaking for 30 min at 25°C for SDS removal. The gels were washed with distilled water for 10 min to remove Triton X-100 and then incubated in 50 mM Tris-HCl (pH 7.5) at room temperature for 24 h. The gels were stained according to standard protocol for electrophoresis. Areas of gelatin hydrolysis were visualized as cleared areas on a Coomassie stained background of the zymograms. Represented zymograms were typical for the series of repeated experiments.

Total proteolytic activity assay. The protease activity was assayed spectrophotometrically according to the method [8]. The reaction was carried out in a reaction mixture containing 50 mM Tris-HCl, pH 7.4, 1% (w/v) casein and 75 µg protein. After 30 min incubation at 37°C the reaction was stopped by adding 9% (v/v) TCA solution followed by 10 min incubation at room temperature and centrifugation (15000 x g, 30 min). The absorbance of the soluble peptides (supernatant) was measured at 280 nm. One unit of the enzyme activity was

defined as the amount of enzyme which releases 1 μmol of tyrosine per min under the assay conditions.

Effect of SDS, Tween 80 and ethanol on protease activity. Protease activity against solvent such as ethanol or detergents (SDS and Tween 80) was determined using casein as substrate. Ethanol (25%, 50%), SDS (1%, 5%), Tween 80 (5%, 20%) was added to the probes containing 75 μg protein and incubated at 37°C with shaking at 150 rpm for 30 min. At the end of the incubation period, residual protease activity was measured by the spectrophotometric method under standard assay conditions (see above) and compared with the activity of the enzyme in the absence of detergent. All the reactions were performed in triplicate.

Results were expressed as means \pm SD. The difference between groups was analyzed by standard Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

Results and discussion. Organic solvents can be advantageous in various industrial enzymatic processes, e.g. the reaction media used in biocatalytic esterification and trans-esterification contains less than 1% water. The use of organic solvents can increase the solubility of non-polar substrates, enhance the thermal stability of enzymes, decrease water-dependent side reactions, or eliminate microbial contamination. For today only few proteases, stable in organic solvent have been purified. Low activity of these enzymes in solvents/detergents can be explained by their partial denaturation, reduced flexibility and limited dispersion of enzymes. Therefore the search for enzymes which are active in the presence of organic solvents or/and detergents will be very useful for biochemical studies as well as for industrial purposes. In recent years, interest in studying of hydrobionts from the Antarctic region has

increased significantly because these organisms are characterized by considerable structural and functional diversity of biologically active compounds, and the presence of enzymes with unique structures and activities. These features, as well as economic profitability, high reproducibility and abundance of marine hydrobionts allowed considering them as a promising source of enzymes for industrial applications, such as the food and detergent industries.

At the first stage of our work we tested crude extract of *A. colbecki* for the enzymes stable in the presence of ionic detergent SDS. For this purpose zymography method was applied. Zymography technique was described as a simple, sensitive, quantifiable and functional assay to analyze active enzymes in biological samples. To assess the enzymatic activity by zymography technique, solution of 12% polyacrylamide can be copolymerized with different high molecular weight substrates, such as starch for identification of amylase activity, and protein substrates, such as gelatin, casein, and collagen, for revealing protease activity. Analyzed extract of *A. colbecki* was submitted to zymography with gelatin (1 mg/ml) as substrate. After electrophoretic separation, the gels were divided into two parts; one part was washed with Triton X-100 and another one used without washing for zymography. It should be noted that at this stage washing in Triton X-100 is necessary for SDS removal. Clear areas on dark background were considered as protease active bands.

In accordance with the results of our experiment, it was found that the proteases of *A. colbecki* able to act in the presence of SDS.

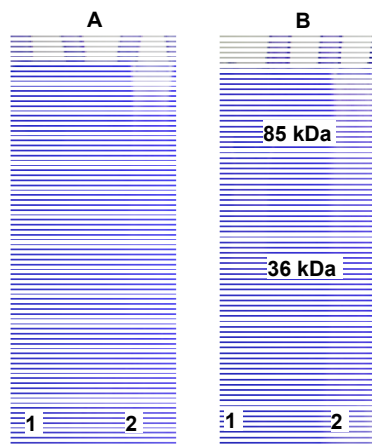


Fig. 1. Typical gelatin zymogram of crude extracts of *A. colbecki* without washing with Triton X-100 (A) and under washing with Triton X-100 (B): 1 – molecular weight markers (36 kDa – miniplasmin, 85 kDa – plasmin); 2 – crude extract of *A. colbecki*

So, we showed that the proteases from crude extracts of *A. colbecki* retained their activities after electrophoresis without washing in Triton X-100 (Fig. 1A). Three clear areas were detected at zymogram in this case. To establish the exact molecular weights of identified proteases zymogram was calculated using TotalLab 2.04 program. According to obtained results the molecular weights of these enzymes were estimated about 24, 40 and 53 kDa. In additional significant proteolytic active zone was found at the top of the track. But after gels washing with Triton X-100 clear bands were more visible and proteolytic activities were localized over the whole length of the track (Fig. 1B).

In contrast, plasmin and miniplasmin used as controls maintained their activity toward gelatin only after washing

gel with Triton X-100 (Fig. 1B). No clear areas were found in the presence of SDS but complete removing of detergent resulted in resumption of enzymatic activity.

Based on obtained results the next goal of our work was to estimate total proteolytic activity in crude extract of *A. colbecki* under incubation with detergents/solvent. As we known detergents are routinely incorporated into protein formulations to solubilize hydrophobic proteins or to act as chaotropic agents to prevent protein aggregation. But their usage in high concentrations may produce protein denaturation by means of their interaction with hydrophobic and hydrophilic regions of the proteins.

Partial characterization of crude extract of *A. colbecki*, without any purification, suggested the

existence of enzymes active in the presence of detergents Tween 80 and SDS. As shown in Table 1 the proteases from crude extract of *A. colbecki* had ability to act at different concentration of Tween 80 in reaction system. So, the incubation with 5% Tween 80 increased total proteolytic activity in extract of *A. colbecki* in 1.2 times whereas under incubation with 20% Tween 80 the proteolytic activity was within the control value. It should

be noted that the incubation of the pure trypsin with Tween 80 at different concentrations caused a significant decrease of enzyme activity. According to obtained results trypsin activity was decreased in 1.8 times compared to the control in the presence of 5% detergent. Higher concentration (20%) of Tween 80 caused a further reduction of proteolytic activity in 2 times.

Table 1. Proteolytic activity under incubation with detergents/solvent

	Trypsin	Crude extract of <i>A. colbecki</i>
	Units/mg protein	
Total proteolytic activity	425.7±6.5	12.8±0.8
5% Tween 80	236.5±3.7*	15.4±1.2*
20% Tween 80	203.5±3.1*	12.3±1.0
1% SDS	no activity	12.2±1.1
5% SDS	no activity	11.8±0.75
25% Ethanol	430.3±5.0	1.56±0.57*
50% Ethanol	239.7±4.2*	no activity

Data are shown as the means ± SD

* – p<0.05 in compare with total proteolytic activity

Enzymes of *A. colbecki* studied in this work maintained stability in the presence of strong ionic surfactant SDS assayed at concentration of 1% and 5% for 30 min. This fact is very interesting and promising because SDS-stable enzymes are not generally available except for some enzymes isolated from microbial source [4]. The total proteolytic activity was not significantly affected by 1% SDS, retaining 95.7% of the initial activity, although total proteolytic activity was decreased slightly (in 1.08 times) when SDS concentration was raised to 5%. Surprisingly, but activity of trypsin in the presence of SDS at different concentrations was found to be strongly inhibited by this detergent. Such result was similar to result of zymography.

So, our results demonstrated stability of the proteases from *A. colbecki* against denaturation or unfolding in the presence of analyzed detergents. The nature of this stability is unclear, but it may be explained by the presence of disulfide bonds which according to [9] are essential for stability of the proteins against detergents. On the other hand detergents can also bind to proteins and induce structural changes that for 5% Tween 80 appear to be stimulatory.

Enzymes stable in organic solvents have been intensively investigated because they have several industrial applications in addition to their using in classic detergent formulations.

Our studies of the stability of *A. colbecki* proteases in ethanol represented at Table 1 too. According to obtained results total proteolytic activity in crude extract of *A. colbecki* decreased in 8.3 times of initial value in case of incubation with 25% ethanol. Further increasing of ethanol concentration to 50% caused a continuous reduction of activity – no proteolytic activity was found at this concentration. Trypsin activity, in contrast, was not strongly inhibited by ethanol. The enzyme was highly stable in the presence of 25% ethanol, showing the retaining of the proteolytic activity at the control level. But preincubation of trypsin with 50% ethanol resulted in decreasing its activity in 1,8 times.

Ethanol-mediated reduction of enzymatic activity may be due to unfolding, structural disfunctioning and stripping of the essential water layer from the enzyme molecule. The presence of organic solvent alters the catalytic process of enzyme by disruption of hydrogen bonds and hydrophobic interactions as well as changes in the dynamics and conformation of the proteins.

Summarizing, the results obtained in this work indicated that the proteases from the Antarctic scallop *Adamussium colbecki* were quite stable in the presence of detergents such as Tween 80, SDS and unstable in the presence of ethanol. Further studies on purification and detail characterization of proteases from *A. colbecki* as well as analysis of other hydrobionts from the Antarctic region on the presence of detergent stable enzymes are necessary and will undoubtedly be carried out.

Acknowledgment

This research was supported by National Antarctic Scientific Center of Ukraine Ministry of Education and Science of Ukraine.

Список використаної літератури

- Bradford M.M. A rapid and sensitive method for quantities of utilizing the principle of protein binding / M.M. Bradford // Analytical Biochemistry. – 1976. – Vol. 86. – P. 193–200.
- Fuchise T. Simple preparation of pacific cod trypsin for enzymatic peptide synthesis / T. Fuchise, H. Sekizaki, H. Kishimura [et al.] // Journal of Amino Acids. – 2014. – Vol. 8. – P. 1–8.
- Gupta A. Purification and characterization of a solvent stable protease from *Pseudomonas aeruginosa* PseA / A. Gupta, I. Roy, S.K. Khare [et al.] // J Chromatogr A. – 2005. – Vol. 1069. – P. 155–61.
- Joo H.S. Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: production and some properties / H.S. Joo, C.G. Kumar, G.C. Park [et al.] // J Appl Microbiol. – 2003. – Vol. 95. – P. 267–72.
- Karan K. Function and biotechnology of extremophilic enzymes in low water activity / K. Karan, M.D. Capes, S. DasSarma // Aquatic Biosystems. – 2012. – Vol. 8. – P. 1–15.
- Klomklao S. Digestive proteinases from marine organisms and their applications / S. Klomklao // Songklanakarin J. Sci. Technology. – 2008. – Vol. 30, № 1. – P. 37–46.
- Li Q. Commercial Proteases: Present and future / Q. Li, L. Yi, P. Marek, B.L. Inverson // FEBS Lett. – 2013. – Vol. 587. – P. 1155–1163.
- Munilla-Moran R. Protein digestion in early turbot larvae, *Scophthalmus maximus* (L.) / R. Munilla-Moran, J.R. Stark // Aquaculture. – 1989. – Vol. 8. – P. 315–327.
- Ogino H. Role of intermolecular disulfide bonds of the organic solvent-stable PST-01 protease in its organic solvent stability / H. Ogino, T. Uchiho, J. Yokoo [et al.] // Applied and Environmental Microbiology. – 2001. – Vol. 67(2). – P. 942–947.
- Ostapchenko L. Enzyme electrophoresis method in analysis of active components of hemostasis system / L. Ostapchenko, O. Savchuk, N. Burlova-Vasilieva // Advances in Bioscience and Biotechnology. – 2011. – Vol. 2. P. 20–26.
- Peck L.S. Extreme sensitivity of biological function to temperature in Antarctic marine species / L.S. Peck, K.E. Webb, D.M. Bailey // Functional Ecology. – 2004. – Vol. 18. – P. 625–630.
- Rai S.K. Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from *Bacillus subtilis* strain DM-04 / S.K. Rai, A.K. Mukherjee // Bioresour. Technol. – 2009. – Vol. 100. – P. 2642–2645.

13. Sawant R. Protease: an enzyme with multiple industrial / R. Sawant, S. Nagendran // World Journal of Pharmacy and Pharmaceutical Sciences. – 2014. – Vol. 3, № 6. – P.568-579.

14. Schiaparella S. A reassessment of the distribution of the common Antarctic scallop *Adamussium colbecki* (Smith, 1902) / S. Schiaparella, K. Linseb // Deep-Sea Research II. – 2006. – Vol. 53. – P. 912–920.

References

1. Bradford MM. A rapid and sensitive method for quantities of utilizing the principle of protein binding. Analytical Biochemistry. 1976;86:193–200.

2. Fuchise T, Sekizaki H, Kishimura H, et al. Simple preparation of pacific cod trypsin for enzymatic peptide synthesis. Journal of Amino Acids. 2014; 8:1–8.

3. Gupta A, Roy I, Khare SK, et al. Purification and characterization of a solvent stable protease from *Pseudomonas aeruginosa* PseA. Journal of Chromatogr A. 2005;1069:155–161.

4. Joo HS, Kumar CG, Park GC, et al. Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: production and some properties. J Appl Microbiol. 2003;95:267–72.

5. Karan K, Capes MD, DasSarma S. Function and biotechnology of extremophilic enzymes in low water activity. Aquatic Biosystems. 2012;8:1-15.

6. Klomklo S. Digestive proteinases from marine organisms and their applications. Songklanakarin Journal of Sci. Technology. 2008;30(1):37–46.

7. Li Q, Yi L, Marek P, Inverson BL. Commercial Proteases: Present and future. FEBS Lett. 2013;587:1155-1163.

8. Munilla-Moran R, Stark JR. Protein digestion in early turbot larvae, *Scophthalmus maximus* (L.). Aquaculture. 1989;8:315–327.

9. Ogino H, Uchiho T, Yokoo J, et al. Role of intermolecular disulfide bonds of the organic solvent-stable PST-01 protease in its organic solvent stability. Applied and Environmental Microbiology. 2001;67(2): 942-947.

10. Ostapchenko L, Savchuk O, Burlova-Vasilieva N. Enzyme electrophoresis method in analysis of active components of hemostasis system. Advances in Bioscience and Biotechnology. 2011;2:20–26.

11. Peck LS, Webb KE, Bailey DM. Extreme sensitivity of biological function to temperature in Antarctic marine species. Functional Ecology. 2004;18:625–630.

12. Rai SK, Mukherjee AK. Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from *Bacillus subtilis* strain DM-04. Bioresour. Technol. 2009;100:2642–2645.

13. Sawant R, Nagendran S. Protease: an enzyme with multiple industrial. World Journal of Pharmacy and Pharmaceutical Sciences. 2014;3(6):568-579.

14. Schiaparella S, Linseb K. A reassessment of the distribution of the common Antarctic scallop *Adamussium colbecki* (Smith, 1902). Deep-Sea Research II. 2006;53:912–920.

Надійшла до редколегії 11.05.16

Н. Ракша, канд. біол. наук, Д. Гладун, асп.
Київський національний університет імені Тараса Шевченка, Київ,

ДЕТЕРГЕНТ-СТІЙКІ ПРОТЕЇНАЗИ АНТАРКТИЧНОГО ГРЕБІНЦЯ *ADAMUSSIUM COLBECKI*

Показано, що екстракт тканин Антарктичного морського гребінця *Adamussium colbecki* містить протеїнази стійкі до дії ряду детергентів. Результати ензим-електрофорезу з використанням як субстрату желатину свідчать про наявність ферментів, що зберігають активність за присутності SDS, в той час як активність плазміну і мініплазміну рееструвалася лише після повного його видалення. Загальна протеолітична активність, яку оцінювали за ступенем гідролізу казеїну після інкубації екстракту тканин *A. colbecki* з детергентами Tween 80 і SDS, зберігалася на рівні контролю, тоді як активність трипсину за аналогічних умов інкубації значно знижувалася.

Ключові слова: морський гідробіонт *Adamussium colbecki*, SDS, Tween 80, етанол, загальна протеолітична активність.

Н. Ракша, канд. біол. наук, Д. Гладун, асп.
Київський національний університет імені Тараса Шевченка, Київ, Україна

ДЕТЕРГЕНТ-УСТОЙЧИВЫЕ ПРОТЕИНАЗЫ АНТАРКТИЧЕСКОГО ГРЕБЕШКА *ADAMUSSIUM COLBECKI*

Показано, что экстракт тканей Антарктического гребешка *Adamussium colbecki* содержит протеиназы устойчивые к действию ряда детергентов. Результаты энзим-электрофореза с использованием как субстрата желатина свидетельствуют о наличии ферментов, сохраняющих активность в присутствии SDS, в то время как плазмин и миниплазмин сохраняли активность только после полного удаления детергента. Общая протеолитическая активность, оцененная по степени гидролиза казеина после инкубации экстракта тканей *A. colbecki* с детергентами Tween 80 и SDS, сохранялась на уровне контроля, тогда как активность трипсина в аналогичных условиях инкубации значительно снижалась.

Ключевые слова: морской гидробионт *Adamussium colbecki*, SDS, Tween 80, этанол, общая протеолитическая активность.

УДК 612.119+616.155.392-036.12+616.15-07

І. Свєженцева, асп., Д. Білько, канд. біол. наук., Н. Білько, д-р мед. наук, проф.
Національний університет "Києво-Могилянська академія", Київ,
І. Дягіль, д-р мед. наук
Державна Установа "Науковий центр радіаційної медицини НАМН України", Київ

ОСОБЛИВОСТІ ПРОЛІФЕРАЦІЇ ТА ДИФЕРЕНЦІЮВАННЯ КЛІТИН-ПОПЕРЕДНИКІВ ЕРИТРОПОЕЗУ ПРИ ХРОНІЧНІЙ МІЄЛОЇДНІЙ ЛЕЙКЕМІЇ В КУЛЬТУРІ КЛІТИН *IN VIVO*

Досліджено особливості проліферації та диференціювання клітин еритроїдної ланки гемопоезу в культурі дифузійних камер *in vivo* у пацієнтів з хронічною мієлоїдною лейкемією при терапії інгібіторами тирозинкіназ – іматинібом та нілотинібом. У результаті культивування було виявлено, що відбувається підвищення проліферативної активності клітин-попередників еритропоезу як у випадку пацієнтів із вперше виявленою лейкемією, так і у зразках кісткового мозку пацієнтів зі стійкістю клітин лейкемічного клону до терапії інгібіторами тирозинкіназ. Продемонстровано пригнічення диференціювання клітин-попередників еритроїдного ряду із набуттям клітинами лейкемічного клону стійкості до інгібіторів тирозинкіназ.

Ключові слова: хронічна мієлоїдна лейкемія, клітини-попередники еритропоезу, культура клітин *in vivo*, інгібітори тирозинкіназ.

Вступ. Хронічна мієлоїдна лейкемія (ХМЛ) вважається одним із найбільш поширених онкогематологічних захворювань [12]. Захворювання характеризується підвищенням проліферації ранніх клітин-попередників мієлоїдної ланки кровотворення, що призводить до накопичення незрілих клітин гранулоцито-макрофагального ряду у кістковому мозку та периферичній крові пацієнтів [2, 3]. Причиною лейкемічної трансформації є поява в ранній гемопоетичній клітині-попереднику дериватної хромосоми 22, яку прийнято називати Філадельфійсь-

кою (Ph-хромосома) [9]. На ній міститься химерний онкоген *bcr-abl*, що кодує однойменний онкобілок, який характеризується тирозинкіназою активністю. BCR-ABL тирозинкіназа активує низку внутрішньоклітинних сигнальних шляхів, призводячи до надмірної проліферативної активності, нечутливості до впливу сигналів кісткоствокового мікрооточення та блокування апоптозу у клітинах лейкемічного клону [7, 10, 11].

Із розвитком більш чутливих модів молекулярної біології було встановлено, що в хронічній фазі захо-