BIOCHEMICAL PROPERTIES OF RECOMBINANT ALKALINE PHOSPHATASE FROM *BACILLUS LICHENIFORMIS* T5

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ABSTRACT

In this work, recombinant alkaline phosphatase (phoB) from *Bacillus licheniformis* was successfully expressed in *Escherichia coli*, purified and biochemically characterized. Gene coding alkaline phosphatase was amplified from genomic DNA of B. licheniformis and cloned into expression vector pET-28c (+). Using the recombinant vector, a BL21 (DE3)/pAlPh strain-producer was obtained with over expression of the gene. Optimal cultivation parameters for producing recombinant alkaline phosphatase have been determined; 10 mg of protein was purified from 1 liter of culture. The activity of the recombinant alkaline phosphatase is 100 U/mg at standard conditions. Biochemical characteristics of recombinant alkaline phosphatase showed that enzyme has maximum activity at pH=10.0 and temperature +60°C. The kinetics of p-nitrophenyl phosphate hydrolysis have been studied, the Michaelis constant Km was 0.91±0.13 mM and the limiting value of the maximal rate of the enzymatic reaction Vmax was 21.4±1.19 mM. Experiments were carried out to determine the dependence of the enzyme activity on various divalent metals.

Keywords: alkaline phosphatase, strain-producer, biochemical characteristics, enzymatic activity, purification, expression, kinetic parameters.

INTRODUCTION

To date, scientific research and diagnostics are actively carried out in Kazakhstan using immunochemical methods, immunoblotting, immunohistochemistry, immunocytochemistry and enzyme immunoassay. Work is under way to develop immunotests for the diagnosis of infectious diseases of medical and veterinary use. However, in the development of tests and in the conduct of research, some components are used from foreign manufacturers. These components are secondary antibodies labeled with enzymes, antibody-binding proteins, enzymes. Accordingly, the actual task is to obtain reagents to complete the developed test systems.

Immunochemical methods of analysis are widely used in various industries, diagnostics, environmental monitoring, food safety. Improvement of immunochemical methods of analysis became possible due to the use of enzymes as labels for the detection of antibody antigen reactions. To detect the catalytic activity of enzymes, a large number of substrates have been proposed in the immunoassay. Most often, substrates are used, which form products absorbing in the visible region of the spectrum. The horseradish peroxidase, β-galactosidase and alkaline phosphatase are used most intensively as labels in the immunoassay.

Alkaline phosphatase is an enzyme that catalyzes the removal of 5'-phosphate groups of DNA or RNA, as well as cleavage of macroergic bonds of deoxyribonucleoside triphosphates [1]. The enzyme catalyzes the hydrolysis of a wide range of phosphomonoester and catalyzes the transphosphorylation reaction by transferring the phosphoryl group to an alcohol in the presence of phosphate acceptors. Isozymes of alkaline phosphatase can split the bound phosphoric acid esters from a wide range of substrates [2].

The practical interest in the enzyme is explained by its wide use in genetic engineering, hybridization DNA technology and in medicine for the production of immunoconjugates and recombinant antibodies. In molecular biology, APh are an effective tool for significantly increasing the efficiency of vector ligation. Dephosphorylating terminal phosphate groups, they prevent self-ligation of the vector [3-5].

A recombinant enzyme with high catalytic efficiency and thermal stability has a high potential for use in clinical diagnostics, where the stability of the enzyme is required against thermal deactivation during preparation or labeling procedures [6].

Since at the present time one of the rapidly developing areas of molecular genetics is protein engineering, which includes the development of protein modification methods for studying their structural and functional interrelations, and also the design of new proteins with specified properties, APh can be a promising target for the production of such recombinant hybrid proteins.

In general, alkaline phosphatase from *Bacillus licheniformis*, which has a high specific activity and a successful combination of other industrially valuable properties, is considered as one of the most promising phosphatases.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and reagents

In this study *Bacillus licheniformis* BT7 was used as a source of genomic DNA for the amplification of the alkaline phosphatase gene. *B. licheniformis* was isolated from the soil of southern region of Kazakhstan. *Escherichia coli* DH5α was used for cloning. For the expression of the heterologous protein following strains of *E. coli* were used: BL21 (DE3), ArcticExpress (DE3) and Rosetta (DE3). The plasmid pET-28c(+) (Novagen, UK)was used to construct the expression vector.

Luria Bertani broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) was used for growth and maintenance of *E.coli* strains. For incubation of the transformed cells SOC medium was used (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 20 mM MgSO4, 20 mM glucose, pH 7.5). The preparation of media was carried out in accordance with Maniatis protocol [7].

All reagents used in this work were manufactured by Sigma-Aldrich, AppliChem, Promega, Amresco with the purity category "For molecular biology".

Construction of the expression vector and cloning in Escherichia coli

The alkaline phosphatase gene *phoB* from genomic DNA of *Bacillus licheniformis* was amplified with specific primers including *Nde*I and *Not*I restriction sites: NdeI-PhoBfw (5'GGAATTCCATATGGGTTTCTTACGCAACAGAAT3') and NotI-PhoBrv (5' ATAGTTTAGCGGCCGCTCTGGCGTATTTTTTGAATAGCT -3'). The PCR conditions for amplification of the target gene were: genomic DNA of *Bacillus licheniformis* (100 ng) 1 µl, (10 µM) of each primers, 4 µl (10 mM total) dNTPs, 10 µl of 5x PCR buffer (containing 7,5 mM MgCl2) and 0,5 µl of Phusion High-Fidelity DNA Polymerase (2 U/µl); firstly PCR mixture was heated for 25 seconds, 30 cycles of subsequent reaction program conducted at 10 sec at +98°C, 20 sec at +55°C, 1 min at +72°C and a final elongation for 7 min at +72°C in the PCR thermocycler Mastercycler nexus gradient (Eppendorf, Germany). The amplified product was analyzed by 1% agarose gel electrophoresis with ethidium bromide. The PCR product was obtained with a corresponding size (~1685 bp). The resulting amplificate was purified by chloroform extraction method and was cloned into vector pET-28c(+) using *Nde*I *and Not*I restriction enzymes. Ligation was performed with T4 DNA ligase at +4°C for 16 hours. The ligation reaction was transformed into DH5α cells plated on LB medium containing 50 µg/mL kanamycin. Total number of colonies was about 50 colonies. Next, PCR screening was performed using T7 (promoter and terminator) primers. Selected colonies-transformants were inoculated in LB broth for producing plasmid DNA. Isolation of plasmids from the positive clones was performed using a kit GeneJet plasmid Miniprep kit (Thermo scientific, USA) according to the manufacturer's protocol. Integrated plasmid insert was sequenced by the T7 primers and tested for compliance with the required sequence of genomic data bank.

Expression of recombinant alkaline phosphatase from Bacillus licheniformis

To determine the optimal strain three strains of *E.coli* with different genotypes: BL21(DE3), ArcticExpress(DE3)RP and Rosetta(DE3) were tested. Transformation of electrocompetent cells by a plasmid vector pET-28c/phoB performed by electroporation using MicroPulser (BioRad, USA) under the following conditions: 100 ng of plasmid per 50 µl cell, voltage – 2,5 kV, electric capacity – 25 µF, resistance – 200 Ohms. Transformed cells were incubated in 950 µl of SOC at +37°C for 1 hour with vigorous shaking. Then 50 µl of cells were plated on LB agar with kanamycin and grown at +37°C for 16 hours. Single colonies-transformants were cultured in LB broth with kanamycin in a volume of 200 ml. In the middle of logarithmic growth phase of the bacterial mass (OD600 = 0,6) inductor – isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0,5 mM and incubated for 16 hours. Collection of cells was performed at +4°C by centrifugation, 6,000 rcf for 7 minutes. Further, SDS-PAGE electrophoresis was performed to determine the recombinant protein expression level.

Purification of the recombinant alkaline phosphatase

Purification of the recombinant alkaline phosphatase was performed from 400 ml of the induced culture. The cells collected by centrifugation (6000×g, +4°C, 7 minutes) were lysed by ultrasonic desintegration (Omni Ruptor 4000 Ultrasonic Homogenizer). Cell lysate was precipitated by centrifugation at 18,200 g, +4°C for 60 minutes. The extract was applied to a HisTrap Chelating HP 1 ml column pre-equilibrated with HisTrap Low Imidasol buffer (500 mM NaCl, 20 mM Hepes-NaOH (pH 7.5), 20 mM Imidazole (pH 7.5). After loading, the column was washed with 5 volumes of HisTrap Low Imidasol buffer to remove nonspecifically bound proteins. To search for the eluting imidazole concentration, a linear imidazole gradient was used using the HisTrap Low Imidasol and HisTrap High

Imidasol buffer (500 mM NaCl, 20 mM Hepes-NaOH (pH 7.5), 500 mM Imidazole (pH 7.5). Fractions containing the recombinant protein, were selected for analysis by SDS-PAGE to check purity. Electrophoretic separation of proteins was performed by the method of Laemmli in 12% polyacrylamide gel under denaturing conditions.

Determination of Alkaline phosphatase activity

The activity of APh was determined by the Bessey-Lowry-Brock method.

The method is based on taking into account the amount of p-nitrophenol, formed as a result of enzymatic cleavage of p-nitrophenyl phosphate (NPP), giving yellow coloration in an alkaline medium. The intensity of the color of the photometric solution is proportional to the activity of the enzyme [8].

The main biochemical indices of recombinant alkaline phosphatase were studied.

The reaction medium consisted of 0.1 M Glycine-NaOH buffer pH=10.0 and dissolved therein nitrophenyl phosphate up to a final concentration of 20 mM. 0.025 ml sample of the enzyme was added into 0.5 ml. of the substrate-buffer mixture. The reaction was carried out for 10 minutes. at + 37°C and stopping with 2.5 ml. 0.1 M NaOH. The intensity of the resulting yellow product was evaluated on a spectrophotometer at a wavelength of 405 nm. Calibration was carried out on nitrophenol. The activity of alkaline phosphatase was estimated by the amount of nitrophenol formed per unit of time.

Dependence of the enzyme activity on the pH of the medium (pH-optimum)

The following conditions were used to determine the pH-dependence of the enzyme: temperature +37°C, exposure time 10 minutes, only pH were changed. The pH of the medium was provided with buffer solutions, 2 types of buffers were used: Tris-HCl for a pH range of 6.5 to 8.5 and Glycine-NaOH for a pH range of 9.0 to 11.5 in increment of 0.5. Nitrophenyl phosphate (NPP) at a concentration of 20 mM in the corresponding buffer was used as the substrate mixture. The substrate mixture for each pH point was verified on a pH-meter and adjusted, if necessary, with 6M HCl or 6M NaOH. The reaction was performed three times with the finding of the arithmetic average of the three tests.

Dependence of the enzyme activity on the temperature of the medium

The temperature dependence of recombinant alkaline phosphatase was studied under the following conditions: Glycine-NaOH buffer at pH=10.0; the final concentration of the substrate – nitrophenyl phosphate (NPP) – 20 mM, only the temperature was changed. All changes in activity were compared with the conditions chosen as standard and were expressed as a percentage: temperature +37°C, pH=10.0, substrate concentration 20 mM. The listed conditions were 100%. The reaction was performed three times for each temperature point with the finding of the arithmetic average of the three tests.

Thermostability of the Alkaline phosphatase

The study of the thermostability of APh was carried out as follows: the enzyme samples were held at different temperatures for 30, 60, 90 and 120 minutes. The enzyme sample was then cooled to room temperature, the enzymatic reaction was carried out under standard conditions (see above). The control was the same enzyme sample without the described temperature effect, which was taken as 100%. The result was expressed as a percentage of the residual activity at each time and temperature point.

The effect of metal ions on the activity of alkaline phosphatase

A study was made of the effect of certain metal ions on the change in the activity of recombinant alkaline phosphatase. The ions of such metals as Co^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Mg^{2+}, were the final concentration in the reaction medium of 0.1 mM, 1 mM and 5 mM, respectively. The conditions for carrying out the biochemical reaction were standard: temperature +37°C, pH=10.0, substrate concentration 20 mM, reaction time 10 minutes.

The kinetic characteristics of the alkaline phosphatase

To determine the kinetic parameters, the following conditions were used: temperature +37°C, exposure time 10 minutes, pH of the medium 10.0; the concentration of the substrate was changed only. The reaction was carried out three times with the finding of the arithmetic average of the three replicates. The initial concentration of the substrate was 0.05 mM and further 0.1; 0.25; 0.5; 0.75; 1.0; 1.5; 2.0; 3.0 mM, respectively. At each point of substrate concentration, activity was found. Then using the Prism 6 software (GraphPad) found the kinetic parameters.

RESULTS AND DISCUSSION

Construction of the expression vector and cloning in Escherichia coli

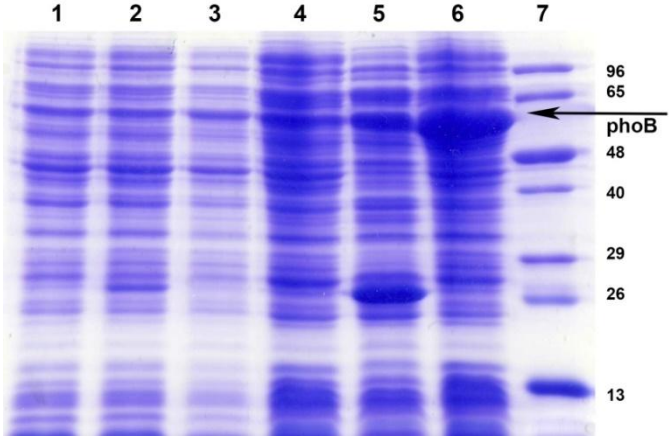
For high-level expression, the pET expression vector system was chosen, which is one of the most powerful systems developed for the expression of recombinant proteins in *E.coli*. Both the DNA fragment and expression vector pET28c(+) were digested with *Nde*I and *Not*I, and the digested DNA fragment was

then inserted into pET-28c(+) to yield the expression plasmid pPhoB. In this vector alkaline phosphatase *phoB* gene is inserted under the control of the T7 promoter. The total length of the protein is 584 amino acid residues with a calculated mass of 63.4 kDa. This sequence contain two hexahistidine (6×His) peptide tags from N and C ends. PCR screening of DH5α colonies-transformants was performed for presence of vector pET-28c with the phoB insert by T7 primers. PCR confirmed the presence of the insert in 5 out of 15 analyzed colonies. Sequencing of plasmid from clone 4 confirmed the absence of mutations in the open reading frame and used for the transformation of *E. coli* BL21(DE3), ArcticExpress(DE3)RP and Rosetta(DE3) cells.

Expression of recombinant alkaline phosphatase from Bacillus licheniformis

E. coli is the most widely used host for production of recombinant proteins. Strain BL21 (DE3) was used in previous studies for alkaline phosphatase production from *B. licheniformis* [9]. In an attempt to increase the yield of purified protein, we also included in this study another DE3 derivative strains, ArcticExpress (DE3) and Rosetta (DE3).

The expression vector pPhoB was transformed to *E. coli* BL21 (DE3), Rosetta (DE3) and ArcticExpress (DE3) for expression of alkaline phosphatase through induction with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside at 37°C when the optical density of OD600=0.6 for 2-16 h. SDS-PAGE analysis of the total cellular proteins separated from the induced cells showed a protein with apparent molecular weight of 63 kDa consistent with the predicted molecular mass of alkaline phosphatase observed in cells transformed with plasmid pPhoB (figure 1).

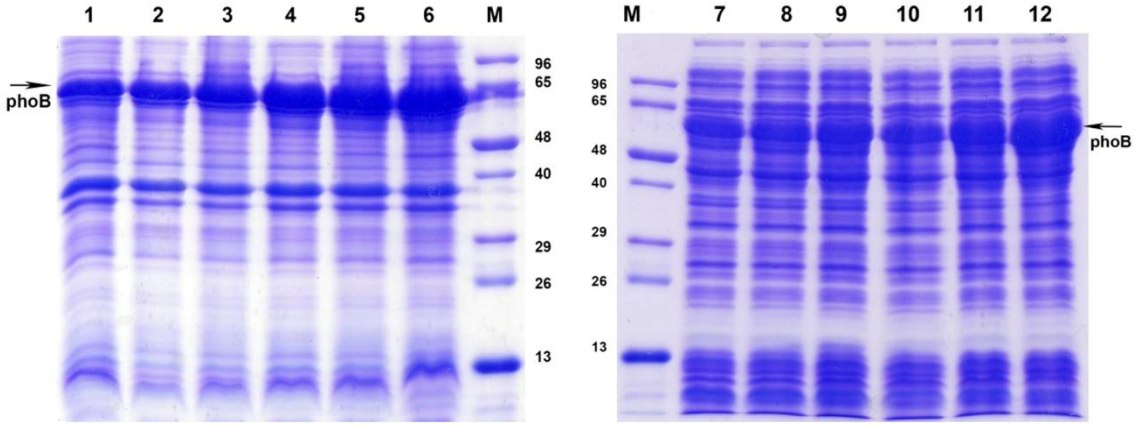


1 – BL21(DE3), 2 – Rosetta(DE3), 3 – ArcticExpress(DE3)RP – 2 hours of induction; 4 – BL21(DE3), 5 – Rosetta(DE3), 6 – ArcticExpress(DE3)RP – 16 hours of induction; 7 – protein marker

Fig. 1. SDS–PAGE analysis of cytosolic (water-soluble) fraction of recombinant alkaline phosphatase

As follows from the results of electrophoresis from (figure 1), all three transformed strains are capable of accumulating in the cells the recombinant protein after 16 hours of induction. But ArcticExpress(DE3) strain has much higher producing ability compared to other, and this strain was used as a producer strain of recombinant alkaline phosphatase.

To improve protein expression in the bacterial strain ArcticExpress (DE3)/pPhoB, we performed induction at a low temperature (18ºC in this study). This strain produces chaperonin Cpn60 and co-chaperonin Cpn10 from the psychrophilic bacterium *Oleispira Antarctica*. Expression vector pPhoB was used for expression of alkaline phosphatase in *E. coli* ArcticExpress (DE3) at 18°C for 1-16 h (figure 2).



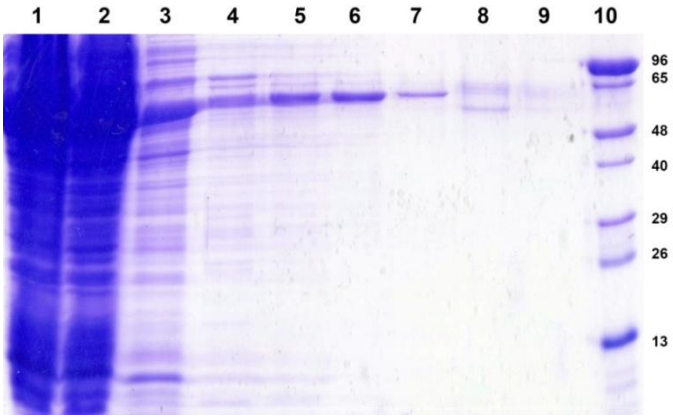
1-6 – pellet; 7-12 – cytosolic (water-soluble) fraction. 1, 7 – 1 hour of induction; 2, 8 – 2 hours of induction; 3, 9 – 4 hours of induction; 4, 10 – 5 hours of induction; 5, 11 – 8 hours of induction; 6, 12 – 16 hours of induction; M – protein marker

Fig. 2. Dependence of the accumulation of recombinant alkaline phosphatase in the cells of ArcticExpress (DE3)/pPhoB on induction time at 18°C

As can be seen from figure 2, the results of the analysis of protein fractions show that the target protein is present in the soluble fraction and in the total lysate consisting of inclusion bodies. The maximum accumulation of the target protein occurs after 16 hours of incubation. Experiments with culturing temperature showed that the recombinant strain have strong temperature dependence. The obtained data suggest the following culture conditions producing strain of recombinant alkaline phosphatase: the cultivation temperature and induction of 37°C…18°C, respectively, under continuous aeration; time incubation of induced culture is 16 hours. These conditions allow obtaining the maximum amount of recombinant protein per unit volume of bacterial culture.

Purification of the recombinant alkaline phosphatase

Recombinant alkaline phosphatase was isolated from the culture of 400 ml by metal affinity chromatography on a column of 1 ml HisTrap Chelaning activated ions Ni^{2+} by a linear gradient of imidazole from 100 mM to 500 mM (figure 3).



1 – loading on the column (load); 2 – fraction through the column (flowthrough); 3 – washing off the column; 4 - 9 – purification fractions; 10 – protein marker

Fig. 3. SDS-PAGE results of purification from the water-soluble fraction

Fractions contain almost pure protein with a molecular weight corresponding to that expected for the recombinant alkaline phosphatase (63 kDa). The yield of alkaline phosphatase phoB was 10 mg from 1 liter of bacterial culture. As a result, 20 mg of recombinant alkaline phosphatase phoB from *Bacillus licheniformis* was produced.

Biochemical characteristic of the enzyme

According to the results of the study, a small activity of APh (less than 2%) from the maximum at pH = 6.5 was recorded. With increasing pH, the activity increased, reaching a maximum at pH = 10.0. After that activity decreased. At pH = 11.5, the activity of the APh was 30% of the maximum (figure 4).

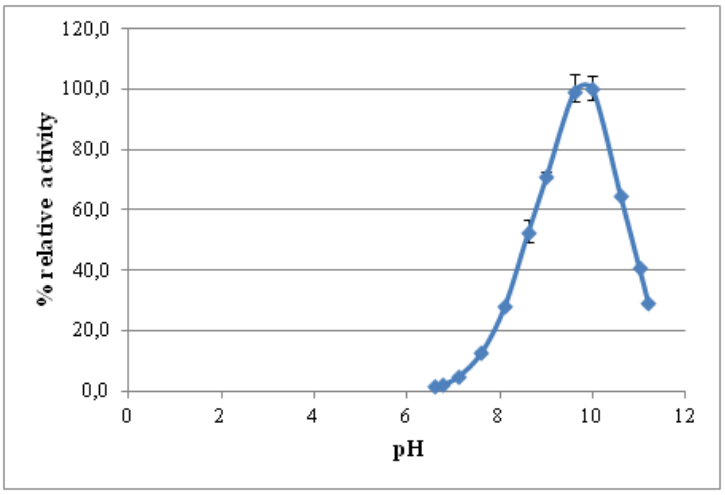


Fig. 4. Phosphatase activity of the phoB enzyme as a function of the pH of the reaction medium

Based on the results of the study at +25°C, the activity of the recombinant APh was almost 85% of the standard. As the temperature of the temperature rises, the activity rises to a maximum at +60°C (almost 185% of the standard one). With further increase in temperature, the activity of the enzyme began to decrease sharply reaching 94% of the standard at +70°C, and about 18% at +90°C. At +95°C, complete inactivation of the enzyme was observed (figure 5).

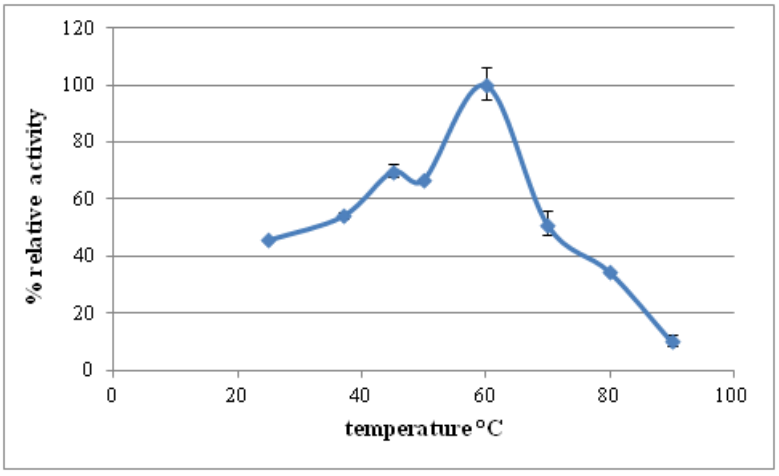


Fig. 5. Phosphatase activity of the phoB enzyme as a function of the temperature of the reaction medium

As a result of the study, it was found that already at +60°C, a fairly rapid inactivation of the enzyme is observed. Thus, at the indicated temperature and 30 minutes exposure, the residual activity of APh is only 24%, after 60 minutes less than 20%, after 90 minutes - slightly more than 12%, and 120 minutes at +60°C - less than 10%. At +70°C, a complete inactivation of the enzyme was observed already at 30 minutes of exposure (figure 6).

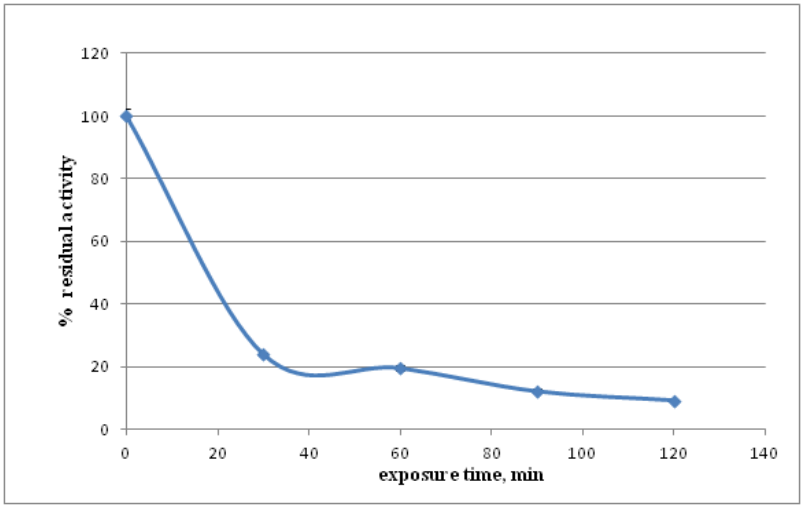


Fig. 6. Dynamics of the dependence of the decrease in the enzymatic activity of recombinant APh from the exposure time at +60°C

A study was made of the effect of certain metal ions on the change in the activity of recombinant alkaline phosphatase. The ions of such metals as Co^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Mg^{2+}, were the final concentration in the reaction medium of 0.1 mM, 1 mM and 5 mM, respectively. The conditions for carrying out the biochemical reaction were standard: temperature +37°C, pH=10.0, substrate concentration 20 mM, reaction time 10 minutes.

Based on the results of the study, the potentiating effect of cobalt ions was noted. Thus, the presence of cobalt ions in all three concentrations almost doubled the reaction rate. Calcium ions inhibited the rate of the reaction dose-dependent. Thus, at a concentration of 0.1 mM, the reaction rate decreased by 6.7%, in the concentration of 1 mM by almost 25%, and in the concentration of 5 mM by almost 38%. Zinc ions had virtually no effect on the reaction rate in any of the concentrations. Cadmium also had no significant effect on the catalytic properties of alkali metals. Magnesium had a moderate potentiating effect by increasing the reaction rate at a concentration of 0.1 mM of about 30%, in a concentration of 1 mM by 24%, in a concentration of 5 mM by 20%.

Based on the results of the study, the substrate concentrations corresponding to the maximum rate V_{max} and half the maximum rate *K*m (Michaelis constant) were established. V_{max}=21.4±1.19 mM/min; *K*m=0.91±0.13 mM (figure 7).

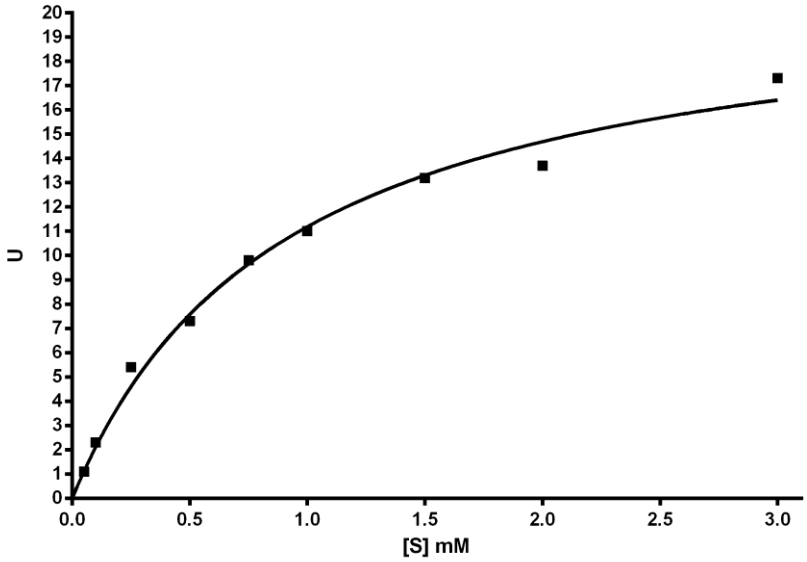


Fig. 7. Kinetic dependence of alkaline phosphatase phoB on substrate concentration

CONCLUSION

In this study, alkaline phosphatase *phoB* gene from *Bacillus licheniformis*, isolated from the soil of southern region of Kazakhstan was inserted into pET-28c(+) vector, over expressed ArcticExpress (DE3),

and its expression was optimized in order to obtain the maximum level of protein production. Histidine tag was added to the protein for easy purification of the enzyme. The expression system and purification method allows to purify a sufficient amount of the recombinant enzyme.

Recombinant alkaline phosphatase phoB shows good thermal stability and catalytic efficiency, has a certain industrial potential, the target protein has a catalytic activity of 100 U/mg of protein. The kinetics of p-nitrophenyl phosphate hydrolysis have been studied, the Michaelis constant K_m was 0.91±0.13 mM and the limiting value of the maximal rate of the enzymatic reaction V_{max} was 21.4 ± 1.19 mM.

Producing strain of recombinant alkaline phosphatase *Escherichia coli*/pPhoB was deposited in the collection of microorganisms "National center for biotechnology", B-NCB 725.

Acknowledgement

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Т5 *BACILLUS LICHENIFORMIS*-ІНІҢ РЕКОМБИНАНТТЫ СІЛТІЛІ ФОСФАТАЗАСЫНЫҢ БИОХИМИЯЛЫҚ СИПАТТАМАЛАРЫ

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ТҮЙІН

Бұл жұмыста *Bacillus licheniformis*-тен алынған рекомбинантты сілтілі фосфатаза (phoB) *Escherichia coli*-ге ойдағыдай экспрессияланып, тазаланып, биохимиялық сипатталған. Сілтілі фосфатазаны кодтаушы ген *B. licheniformis*-тің геномдық ДНҚ-сынан бөліп алып, pET-28c(+) экспрессиялық вектор құрамында көбейтілді. Осы рекомбинантты векторды қолданып рекомбинантты ақуыз көп экспрессияланатын BL21 (DE3)/pAlPh штамм-продуценті алынды. Рекомбинантты сілтілі фосфатаза алуға арналған өсірудің оңтайлы параметрлері анықталды. Ақуыз шығымы 1 литр дақылдан 10 мг-ды құрады. Рекомбинантты сілтілі фосфатазаның белсенділігі қалыпты жағдайларда 100 бірлік/мг-ды құрады. Рекомбинантты сілтілі фосфатазаның биохимиялық сипаттамалары фермент pH=10,0 және температура +60°C жағдайында барынша белсенділікке ие екенін көрсетті. п-нитрофенилфосфат ыдырауының кинетикасы зерттелді, Михаэлис тұрақтысы K_m 0,91±0,13 мМ-ді құрады, ал ферменттік реакцияның алғашқы жылдамдығының мағынасы V_{max} 21,4±1,19 мМ/мин-ті құрады. Фермент белсенділігінің әртүрлі қосвалентті металдарға тәуелділігін анықтау бойынша эксперименттер жүргізілді.

Негізгі сөздер: сілтілі фосфатаза, штамм-продуцент, биохимиялық сипаттамалар, ферменттік белсенділік, тазалау, экспрессия, кинетикалық параметрлер.