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Isolation and Purification of Recombinant Serine/Threonine Protein Kinases of the Strain *Bifidobacterium longum* B379M and Investigation of Their Activity

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Abstract—Previously, we identified six serine/threonine protein kinases (STPK) of *Bifidobacterium* and named them Pkb1–Pkb6. In the present study, we optimized methods for isolation of the six STPK catalytic domains proteins of *B. longum* B379M: a method for isolation of Pkb3 and Pkb4 in native conditions, a method for isolation of Pkb5 in denaturing conditions, and a method for isolation of Pkb1, Pkb2, and Pkb6 from inclusion bodies. The dialysis conditions for the renaturation of the proteins were optimized. All of the enzymes were isolated in quantities sufficient for study of the protein activity. The proteins were homogeneous according to SDS-PAGE. The autophosphorylation ability of Pkb1, Pkb3, Pkb4, and Pkb6 was investigated for the first time. Autophosphorylation was detected only for the Pkb3 catalytic domain.

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Key words: serine/threonine protein kinases, protein isolation and purification, autophosphorylation

Serine/threonine protein kinases (STPK) (EC 2.7.11.1) are enzymes that modify other proteins by reversible phosphorylation at serine or threonine residues within consensus sequences, thereby modulating the activity of a broad range of enzymes and controlling specific protein–protein interactions. Eukaryotic-type STPKs are ubiquitously distributed among Gram-positive bacteria [1]. These enzymes are found in soil microorganisms, pathogens, commensals, and probiotic bacteria [2]. Some bacterial genera such as *Lactobacillus*, *Staphylococcus*, *Streptococcus*, and *Bacillus* express one or two STPKs [2]. The highest number of STPKs was found in the class Actinobacteria: >10 in genus *Mycobacterium* [1], >20 in genus *Nocardia* [2], >30 in genus *Streptomyces* [3], and six STPKs were identified in genus *Bifidobacterium* [4].

Abbreviations: a.a., amino acid residue; DTT, dithiothreitol; IPTG, isopropyl β-D-1-thiogalactopyranoside; PASTA, penicillin-binding protein- and serine/threonine kinase-associated domain (located in the *C*-terminal region of some bacterial proteins); PASTA_pknB, PASTA domain of bacterial PknB-like serine/threonine protein kinases; PMSF, phenylmethylsulfonyl fluoride; STPK, serine/threonine protein kinase; TM-region, transmembrane region.

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Reversible phosphorylation is one of the most important tools of signals transduced from the environment into bacterial cells as well as in regulating cellular functions [5-7]. STPKs and coupled serine/threonine phosphatases regulate expression of prokaryotic genes via posttranslational modification of various proteins including components of the transcription and translation systems [8, 9]. It was demonstrated that STPKs play essential role in cell growth and cell division, DNA replication [10], cell wall biosynthesis [11], biofilm production [12], sporulation and spore germination [13], virulence [5], and stress response and adaptive response [14, 15]. Inhibitors of STPKs are considered as promising antimicrobial agents [16, 17].

In recent years, increased interest has been given to examining bifidobacteria based on their probiotic properties and ability to positively influence human health [18]. Bifidobacterial strains are widely used in the food industry for obtaining fermentable nutritional and medicinal products with probiotic activity [19]. Bifidobacteria are one of the most important representatives of human microbiota, which are dominant in the gut microbiota in infants [20, 21]. Recently, a role of bifidobacteria in the "gut microbiota–brain axis" has been attracting major interest, primarily in the process of the developing nervous system and brain in children [22-25].

Until recently, data regarding examination of structure and functions exhibited by bifidobacterial STPKs were unavailable both in Russian and foreign publications. It should be noted that the experimental data on the functions of bifidobacterial genes are extremely limited because genetic manipulation systems, especially efficient methods for targeted gene mutagenesis, have not yet been established [26]. Functions of almost half of the proteins were only predicted by *in silico* analysis, based on homology with known proteins of other bacteria, and the others are still hypothetical proteins [27]. Similarly to other actinobacteria, it is plausible to suppose that STPK of *Bifidobacterium* can be elements of signal transduction networks and play major regulatory roles in bifidobacterial cells.

Earlier, we identified and characterized six STPKs in various strains of *B. longum* [4, 28]. Because investigation of functions of bifidobacterial protein kinases via the gene knockout is inaccessible due to low efficiency of transformation and recombination [26, 27], direct study of properties and functions of these protein kinases, search for their substrates, and determination of 3D-crystal structures is needed. In this connection, the question of obtaining target proteins in amounts sufficient for preparative analysis is raised.

The aim of the current study was to develop methods for isolation of protein kinases Pkb1-Pkb6 derived from strain *B. longum* B379M [29] and to determine the ability of protein kinases Pkb1, Pkb3, Pkb4, and Pkb6 for autophosphorylation *in vitro*.

MATERIALS AND METHODS

Bacterial strains, vectors, media, and culture conditions. The following strains of *Escherichia coli* were used in the study – BL21(DE3): *F⁻ ompT hsdSB* (*rB⁻mB⁻*) *gal dcm* (*DE3*) (Novagen, USA) [30], and BL21(DE3) pLysS: *F⁻ ompT hsdSB* (*rB⁻mB⁻*) *gal dcm* (*DE3*) [pLysS Cam^R] (Stratagene, USA) [31], plasmid pET32a (Novagen) [30] and plasmids pET32a:*pkb1*, pET32a:*pkb2*, pET32a:*pkb3*, pET32a:*pkb4*, pET32a:*pkb5*, and pET32a:*pkb6* containing the cloned catalytic domains of serine/threonine protein kinases of the industrial probiotic strain *B. longum* B379M [4, 29].

Luria media (L-broth) and TB medium with chloramphenicol (34 μg/ml) were used to grow *E. coli*. Solid media contained 2% agar [32]. Selective growth of plasmid-containing bacteria was provided by adding ampicillin (150 μg/ml). Isolation of plasmid DNA, preparation of competent *E. coli* culture, and transformation were done according to standard methods [32].

Production of *E. coli* biomass for isolation of recombinant protein kinases' catalytic domains. *Escherichia coli* containing recombinant plasmids were grown in a shaker incubator at 37°C until reaching optical density 0.6 (~2 h) followed by inducing expression of STPK genes by adding IPTG. After that, the *E. coli* was cultured at 28°C for 5 or 18 h followed by collection of biomass. The bacteria were sedimented by centrifuging (5000 rpm, 10 min, 4°C) and frozen at –20°C. To analyze expression, cell pellet was suspended in sample buffer (62.5 mM Tris-HCl, pH 6.8, 5% glycerol (w/v), 2% 2-mercaptoethanol, 0.1% SDS, 0.001% bromophenol blue) followed by heat-degradation at 95°C for 10 min. The protein fraction was analyzed by SDS-PAGE. The protein fraction obtained from *E. coli* strains containing insertion-free plasmid pET32a was used as a control sample.

Isolation of recombinant protein kinases. Recombinant proteins from *E. coli* lysates containing recombinant plasmids were isolated according to the QIAexpress protocol in native or denaturing conditions chromatographically by using the Ni-NTA Fast Start Kit (6) (Qiagen, USA). Protein fractions were analyzed by SDS-PAGE. Concentration of purified proteins was quantified according to the Bradford method [33]. Solutions of the isolated proteins were supplemented with glycerol (final concentration – 20%) and kept at –20°C.

Mass-spectrometric assay of purified proteins. The analysis was performed at the Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences. In-PAGE trypsinolysis for further analysis was done using peptide mass fingerprinting as earlier described [34]. Mass-spectrometry assay of tryptic hydrolysates was done by applying MALDI TOF on an Ultraflex mass spectrometer (Bruker Daltonics, Germany). Positively charged ions were quantified in reflection mode ranging within *m/z* 600-4000 Da. Examined proteins were identified using the Mascot search engine (MatrixScience) (http://www.matrixscience.com/home.html). The following search parameters were applied: Enzyme – Trypsin; Database – SwissProt; Taxonomy – Actinobacterium; Fixed modification – none; Variable modification – none; Missed cleavages – 1; Peptide tolerance – 100 ppm; Mass values (monoisotopic) – MH^+.

***In vitro* quantified autophosphorylation of recombinant protein kinases.** The autophosphorylation reaction was performed for 30 min at 37°C in buffer containing 25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, 1 mM EDTA, and 1 mM PMSF. The reaction was triggered by adding ATP to final concentration 10 μM (10 μCi [γ-^{32}P]ATP; Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences), in the presence of 1 μg protein kinase. The reaction was stopped by adding SDS-PAGE sample buffer. Then, the reaction mixture was heated for 5 min at 95°C, followed by analyzing the samples by running 12.5% SDS-PAGE. For autoradiography, the gel was fixed in 50% TCA, stained with 0.1% Coomassie R250 dye, and exposed to KODAK MXG X-ray film.

In vitro autophosphorylation of protein kinases was also done using Kinase-Glo® Plus Luminescent Kinase

Assay V3772 (Promega, USA). The degree of autophosphorylation was determined by quantifying the amount of ATP remaining in solution following the kinase reaction. Kinase-Glo reagent utilizing ATP as a substrate for Ultra-Glo-luciferase catalyzes luciferin oxidation accompanied by emission of photons. The activity of autophosphorylation was inversely proportional to the intensity of the luminescent signal. The autophosphorylation reaction was performed for 30 min at room temperature in buffer containing 15 mM HEPES-NaOH (pH 7.4), 20 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.02% Tween 20, and 0.01% BSA. The reaction was triggered by adding ATP (final concentration 10 μM) in the presence of 5, 10, or 20 μg of the protein kinases, and it was stopped by adding an equal volume of Kinase-Glo reagent. The luminescence signal induced during the reaction was quantified using the DTX 880 Multimode Detector (Beckman Coulter, USA) for 100 msec with sensitivity mode expected high activity.

Bioinformatic analysis methods. Nucleotide and amino acid sequences were searched using databases available at the NCBI (http://www.ncbi.nlm.nih.gov/) and UniProt (http://www.uniprot.org/) websites.

Molecular weight and protein isoelectric points were calculated using ProtParam (http://web.expasy.org/protparam/) software.

RESULTS AND DISCUSSION

Main characteristics of genes and serine/threonine protein kinases of strain *B. longum* B379M. The genome of *B. longum* subsp. *longum* contains six genes encoding STPKs: four conservative genes (*pkb1*, *pkb3*, *pkb5*, and *pkb6*), one species-specific gene (*pkb2*), and one unique gene (*pkb4*) (Table 1) [4, 28]. The *pkb5* and *pkb6* genes are partially overlapped and form an operon. Based on high similarity of amino acid sequences of catalytic domains from bifidobacterial STPKs Pkb5 and Pkb6 as well as protein kinases PknB and PknA from *Mycobacterium tuberculosis*, and given a high similarity in the genetic environment around *pknB-pknA* and *pkb5-pkb6*, it can be assumed that he functions of these protein orthologs are similar and might be related to growth and division of the bacteria [4]. Gene *pkb1* is located close to gene *plsC1* responsible for synthesis of membrane phospholipids as well as genes coding for σ-factor of RNA polymerase and DNA-gyrase. A gene involved in signal transduction is located near the *pkb3* gene.

The genetic environment *pkb2* (the gene encoding the domain of fibronectin and cytokine receptor; gene encoding signaling FHA-domain) as well as unusually high divergence of amino acid sequence of the catalytic domain in Pkb2 from various species of bifidobacteria suggest that this gene might be involved in their adaptation to environment. The unique gene *pkb4* typical to species *B. longum* and *B. bifidum* is located next to the gene encoding the DNA-binding protein belonging to the family of transcriptional regulators involved in adaptive cell reactions [4, 28].

Earlier, catalytic domains from all six protein kinases Pkb1-Pkb6 found in strain *B. longum* B379M we cloned in *E. coli* within expression vector pET32a [4].

Optimization of conditions for culturing *E. coli* strains containing recombinant STPKs. Recombinant strains of *E. coli* containing plasmids pET32a:*pkb1*, pET32a:*pkb2*, pET32a:*pkb3*, pET32a:*pkb4*, pET32a:*pkb5*, and pET32a:*pkb6* were grown in LB- and TB-culture media

Table 1. Characteristics of serine/threonine protein kinases of *B. longum* subsp. *longum*

STPK	Characteristics of full-size (catalytic domain) STPK				Characteristics of *C*-terminal region
	gene length, bp	protein length, a.a.	Mr, kDa	p*I*	
Pkb1	2274 (726)	757 (242)	80.070 (26.334)	5.14 (5.74)	3 PASTA domains, 2 PASTA_pknB2 domains, 1 TM region
Pkb2	1422 (732)	473 (244)	51.540 (26.766)	6.32 (6.47)	1 TM region
Pkb3	2220 (861)	739 (287)	76.989 (30.363)	4.87 (4.88)	5 TM regions
Pkb4	1701 (708)	566 (236)	60.439 (25.916)	5.34 (5.98)	1 TM region
Pkb5	2073 (804)	690 (268)	72.260 (29.344)	5.54 (5.59)	4 PASTA_pknB domains, 1 TM region
Pkb6	951 (735)	316 (245)	34.592 (26.705)	6.97 (5.68)	–

Note: The data presented in the table match those from sequenced genomes from all strains of *B. longum* subsp. *longum* (http://www.ncbi.nlm.nih.gov/genome/?term=Bifidobacterium%20longum).

followed by induction for gene expression by adding IPTG at concentrations of 0.25, 0.5, 1.0, and 1.5 mM for 5 or 18 h. After that, the bacteria were sedimented by centrifuging (5000 rpm, 10 min, 4°C), resuspended in sample buffer, and analyzed by SDS-PAGE.

The maximum expression of all protein kinases was observed by using 1 mM IPTG applied during 18-h culture in TB-medium. The expression level of the target proteins comprised as much as 40-50% of the total cell protein. By analyzing electrophoregrams, it was found that it resulted in expression of proteins with Mr 44, 45, 48, 44, 47, and 45 kDa, which corresponded to the expected molecular weights of recombinant proteins of the catalytic domains from protein kinases summed with Mr matching the protein of the entire linker plasmid pET32a containing the thioredoxin gene (*Trx*). Biomass yield from the 15-ml cell culture was centrifuged (6000 rpm (4226*g*), 15 min, 4°C) and frozen to –20°C for subsequent isolation of the proteins.

Development of conditions for lysis of *E. coli* cells for isolation of catalytic domains of protein kinases from *B. longum* B379M. To check the ability to isolate native proteins derived from *E. coli* containing recombinant plasmids, the bacteria were thawed at 4°C, resuspended in 3 ml of native lysis buffer (50 mM NaH_2PO_4, 5 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF, 5 mM DTT, pH 8.0) supplemented with lysozyme (1 mg/ml) and 20 mM 2-mercaptoethanol, and incubated at 4°C for 1 h. Then the cells were sonicated for 4 min, avoiding warming the suspension, and centrifuged (7500 rpm (6600*g*), 30 min, 4°C) for separating soluble fraction (lysate) and pellet (inclusion bodies). The final fractions were analyzed by running SDS-PAGE (Fig. 1). Based on the lysis data, strain *E. coli* BL21(DE3)pLysS was selected for further experiments.

Analysis of isolated fractions containing Pkb3 and Pkb4 proteins revealed that the target proteins were found both in the soluble fraction and in the inclusion bodies, at approximately equal ratio, thereby providing the opportunity to isolate these proteins under native conditions. Examining fractions of the isolated proteins Pkb1, Pkb2, Pkb5, and Pkb6 showed that the target protein was entirely found in the pellet (enriched in cells in the form of insoluble inclusion bodies), and these proteins might be isolated only under denaturing conditions with subsequent protein refolding.

Isolation of catalytic domains of protein kinases Pkb3 and Pkb4 from soluble fraction. To increase solubility of the target proteins Pkb3 and Pkb4, conditions used for culturing strains *E. coli* containing recombinant plasmids pET32a:*pkb3* and pET32a:*pkb4* were modified: induction of IPTG-triggered expression was done on lowering temperature from 28 to 26°C, and propagation time was shortened to 5 h. Under these conditions, more than 70% of the target protein was in the soluble fraction.

Strains of *E. coli* containing recombinant plasmids were cultured under optimized conditions in 250 ml of culture medium. Proteins were isolated using the Ni-NTA Fast Start Kit (6). All consequent steps of protein isolation were performed at 4°C. The pellet was resuspended in 15 ml of native lysis buffer supplemented with lysozyme and 2-mercaptoethanol and incubated for 1 h at 4°C. Then, the fraction was sonicated for 4 min avoiding warming of the suspension, and centrifuged (7500 rpm (6600*g*), 30 min, 4°C) to sediment cell debris. The lysates were placed on a column pre-equilibrated with lysis buffer: for this, the column was washed three times with washing buffer (50 mM NaH_2PO_4, 5 mM Tris-HCl, 300 mM NaCl, 50 mM imidazole, 1 mM PMSF, 5 mM DTT, pH 8.0). Bound proteins were twice eluted from the column by applying 300 mM imidazole-containing buffer.

Electrophoregram of stepwise isolation and purification of catalytic domain from protein kinase Pkb3 is

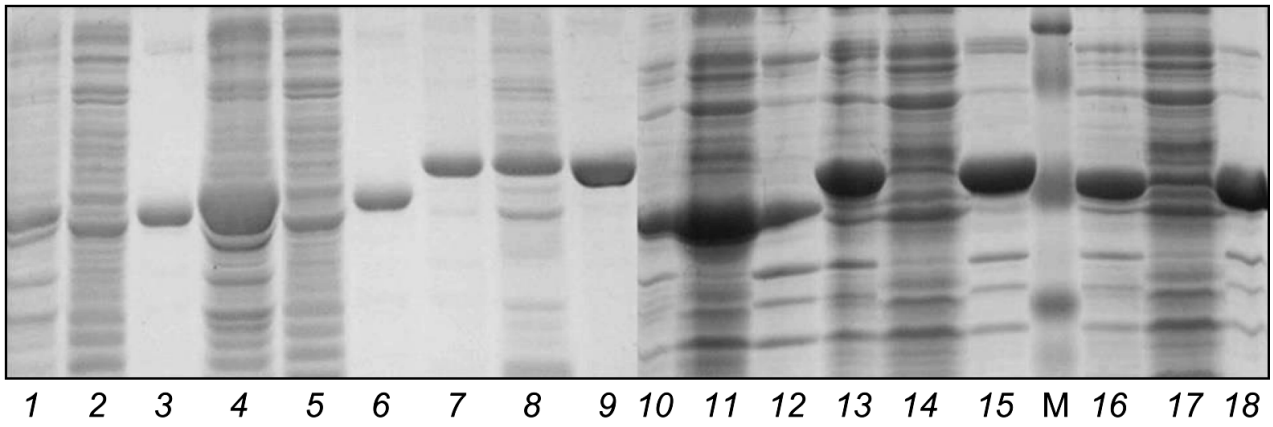

Fig. 1. Electrophoregram of protein fractions from *E. coli* BL21(DE3)pLysS containing plasmids pET32a:*pkb1*-pET32a:*pkb6*. Lanes: *1-3*) Pkb1; *4-6*) Pkb2; *7-9*) Pkb3; *10-12*) Pkb4; *13-15*) Pkb5; *16-18*) Pkb6; *1*, *4*, *7*, *10*, *13*, *16*) control samples; *2*, *5*, *8*, *11*, *14*, *17*) lysates; *3*, *6*, *9*, *12*, *15*, *18*) pellets (inclusion bodies); M, molecular weight protein marker SM0441 (Fermentas, Lithuania).

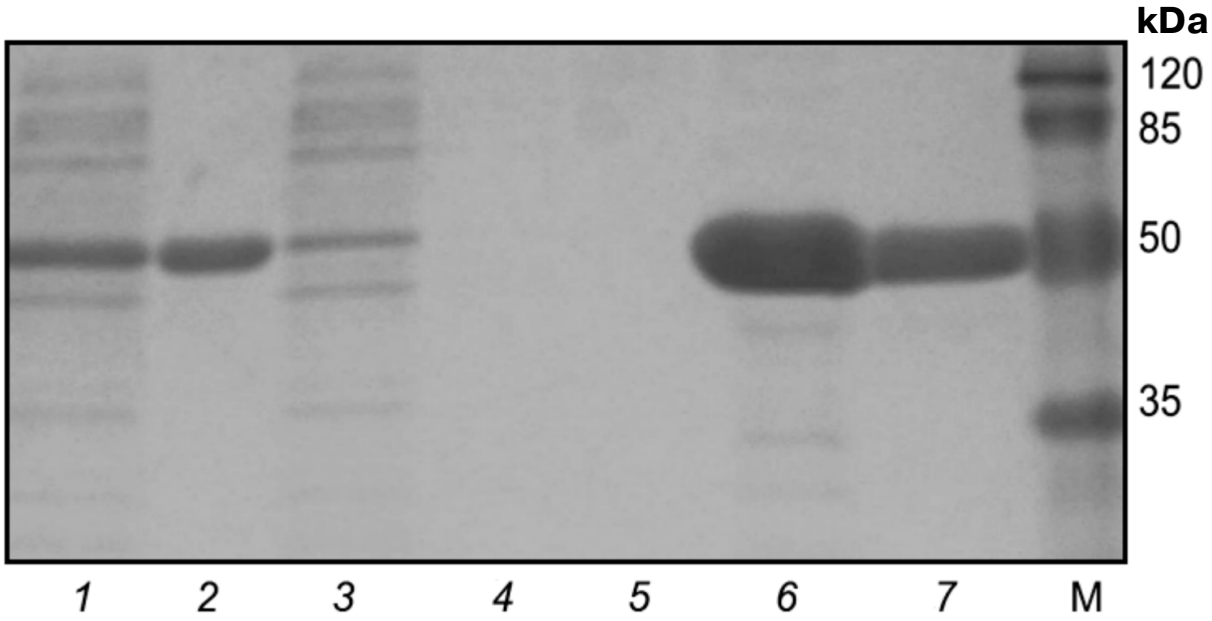

Fig. 2. Electrophoregram of stepwise isolation and purification of catalytic domain from protein kinase Pkb3. Lanes: *1*) lysate; *2*) pellet; *3*) flow-through; *4*) wash-1; *5*) wash-2; *6*) eluate-1; *7*) eluate-2; M, molecular weight protein marker SM0441 (Fermentas, Lithuania).

shown on Fig. 2. A 250-ml culture medium yielded 11.6 ± 1.5 and 28 ± 3 mg of Pkb3 and Pkb4, respectively, with purity ⩾98%. Mass-spectrometric analysis confirmed that the isolated proteins were catalytic domains of protein kinase Pkb3 and Pkb4 derived from *B. longum*.

Isolation of catalytic domains of protein kinases Pkb1, Pkb2, Pkb5, and Pkb6 from insoluble fraction. The target proteins were isolated under denaturing conditions. For refining conditions for lysis of strains of *E. coli* containing recombinant plasmids pET32a:*pkb1*, pET32a:*pkb2*, pET32a:*pkb5*, and pET32a:*pkb6*, the bacteria were grown being induced with 1 mM IPTG in 15 ml TB-medium containing chloramphenicol and ampicillin. After that, the bacteria were centrifuged (5000 rpm (2935*g*), 10 min, 4°C), resuspended in 3 ml of denaturing lysis buffer (8 M urea, 100 mM Na_2HPO_4, 10 mM Tris-HCl, pH 8.0), and incubated at room temperature for 1 h. However, after separating the final lysate into soluble and insoluble fractions, all target proteins were found in the pellet, thereby significantly hindering their further purification and renaturation. The simplest way to increase efficacy of the lysis procedure was to extend exposure time of lysis up to ⩾3 h followed by sonication. Under these conditions, the target proteins went into the soluble fraction without binding to Ni-agarose column. Owing to this, the concentration of urea was lowered to 6 M in all buffer solutions. Cells centrifuged from 250-ml culture medium were resuspended in 15 ml of denaturing lysis buffer (6 M urea, 100 mM Na_2HPO_4, 10 mM Tris-HCl, pH 8.0) and incubated at room temperature for 2.5 h followed by sonication for 4 min and centrifugation (7500 rpm (6600*g*), 30 min, 4°C). Then the lysate was placed on a column pre-equilibrated with lysis buffer containing 6 M urea. The columns were washed three times with denaturing wash buffer (6 M urea, 100 mM Na_2HPO_4, 10 mM Tris-HCl, 50 mM imidazole, pH 5.3). Bound proteins were eluted twice from the columns using denaturing buffer containing 6 M urea and 300 mM imidazole.

A pure catalytic domain of protein kinase Pkb5 was isolated under the above noted conditions. After that, the purified protein was restored using 2-mercaptoethanol and renatured by dialyzing against buffer (1.5 M urea, 50 mM Tris-HCl, 200 mM NaCl, 10% glycerol, pH 8.0). The yield of Pkb5 from 250 ml of culture medium was 10 ± 1 mg, purity ⩾98% (Fig. 3). Mass-spectrometric analysis confirmed that the isolated protein was the catalytic domain of protein kinase Pkb5 derived from *B. longum*.

Catalytic domains of protein kinase Pkb1, Pkb2, and Pkb6 failed to be isolated under denaturing conditions by applying the method used to isolate Pkb5. Therefore, another method was developed to isolate Pkb1, Pkb2, and Pkb6 from inclusion bodies. In particular, the bacteria were resuspended in 15 ml of lysis buffer supplemented with lysozyme, 2-mercaptoethanol, and 0.3% Triton X-100 (to increase solubility) for 1 h followed by 4-min sonication and centrifugation (7500 rpm (6600*g*), 30 min, 4°C). The inclusion bodies were twice washed with 10 ml of lysis buffer supplemented with 0.5% Triton X-100, sonicated for 2 min, and centrifuged (7500 rpm (6600*g*), 30 min, 4°C). Then the inclusion bodies were resuspended in 15 ml of denaturing lysis buffer supplemented with 6 M urea, incubated at room temperature for 30 min, and centrifuged (see above), and the lysate was applied to a

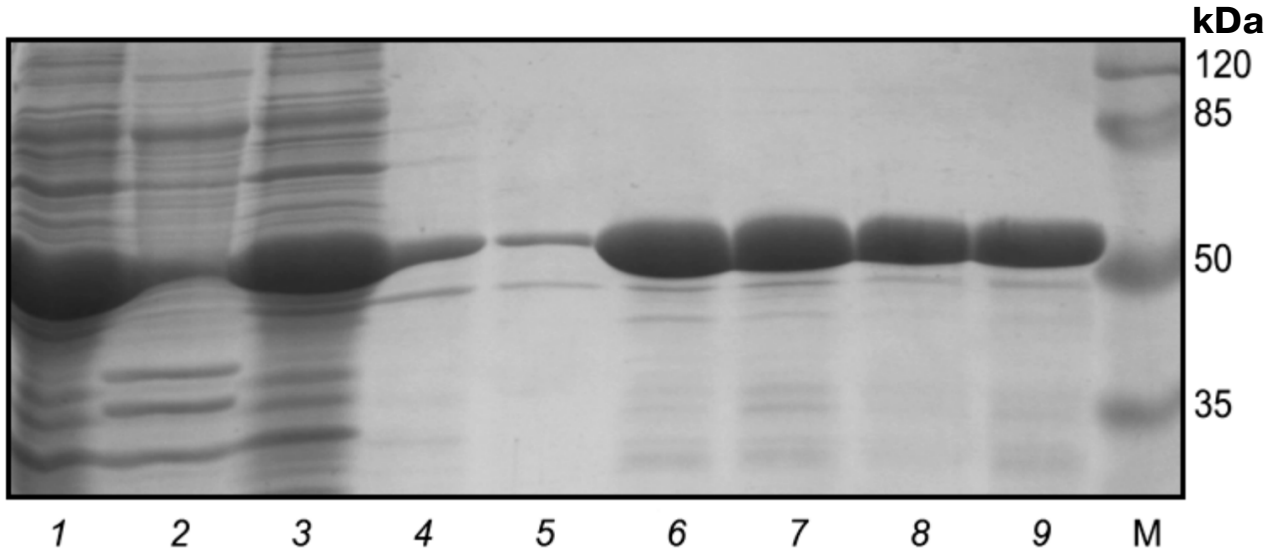

Fig. 3. Electrophoregram of stepwise isolation and purification of catalytic domain from protein kinase Pkb5 under denaturing conditions. Lanes: *1*) lysate; *2*) pellet; *3*) flow-through; *4*) wash-1; *5*) wash-2; *6*) eluate-1; *7*) eluate-2; *8*) eluate-3; *9*) post-dialysis sample; M, molecular weight protein marker SM0441 (Fermentas, Lithuania).

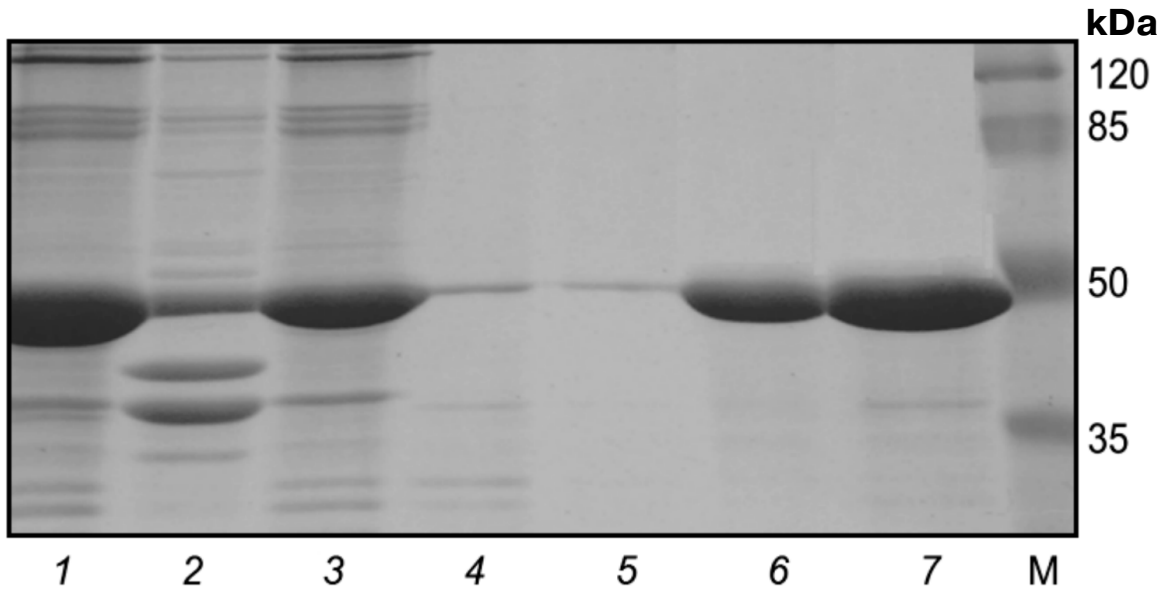

Fig. 4. Electrophoregram of stepwise isolation and purification of catalytic domain from protein kinase Pkb2 derived from inclusion bodies. Lanes: *1*) pellet; *2*) lysate; *3*) flow-through; *4*) wash-1; *5*) wash-2; *6*) eluate-1; *7*) eluate-2; M, molecular weight protein marker SM0441 (Fermentas, Lithuania).

column pre-equilibrated with denaturing lysis buffer. The column was washed three times with denaturing wash buffer (6 M urea, 100 mM Na_2HPO_4, 10 mM Tris-HCl, 50 mM imidazole, pH 5.3). Bound proteins were eluted twice from the column using denaturing buffer containing 6 M urea and 300 mM imidazole. Then the target proteins were reduced using 2-mercaptoethanol and renatured by dialysis (see above).

Electrophoregram of stepwise isolation and purification of catalytic domain from protein kinase Pkb2 is shown on Fig. 4. The yield of Pkb1, Pkb2, and Pkb6 obtained from 250-ml culture media was 8.0 ± 1.0, 8.5 ± 1.3, and 8.5 ± 1.8 mg, respectively, with purity ⩾98%. Mass-spectrometric analysis confirmed that the isolated proteins were the catalytic domains of protein kinases Pkb1, Pkb2, and Pkb6 of *B. longum*.

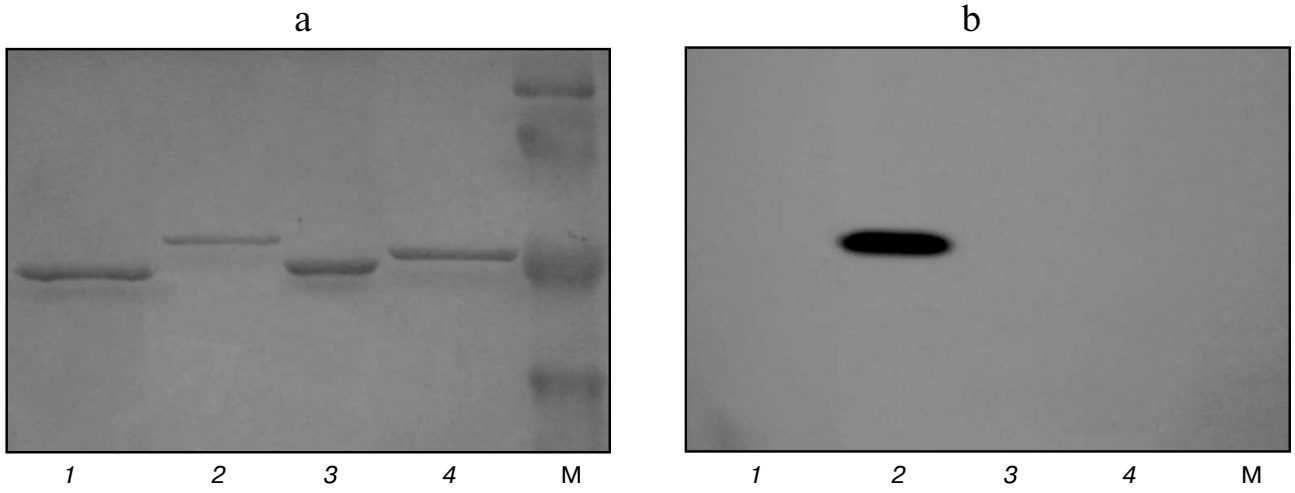

Fig. 5. Autophosphorylation of catalytic domains of protein kinases quantified by using [γ-^{32}P]ATP. a) Electrophoregram of autophosphorylated protein kinases: *1*) Pkb1; *2*) Pkb3; *3*) Pkb4; *4*) Pkb6; M, molecular weight protein marker SM0441 (Fermentas, Lithuania). b) Autoradiograph of autophosphorylated protein kinases.

Investigation of autophosphorylation of isolated proteins Pkb1, Pkb3, Pkb4, and Pkb6. The autophosphorylation reaction is the first step in functioning of many bacterial STPKs [35-37]. Earlier, we demonstrated that two STPKs (Pkb2 and Pkb5) [4] of the six kinases examined in the current study are capable of autophosphorylation. Here, we investigated the potential for autophosphorylation in the four remaining STPKs by applying two approaches: using [γ-^{32}P]ATP and the luminescence reaction. In particular, proteins were incubated with [γ-^{32}P]ATP in buffer solution followed by SDS-PAGE (Fig. 5a). To detect radiolabeled proteins, the SDS-PAGE gel was exposed to X-ray KODAK MXG film (100sh) (Fig. 5b).

The data demonstrate that the catalytic domain of protein kinase Pkb3 is capable of autophosphorylation; however, the catalytic domains from Pkb1, Pkb4, and Pkb6 were found to lack the ability to autophosphorylate under these conditions.

Autophosphorylation of proteins Pkb1, Pkb3, Pkb4, and Pkb6 was also examined by measuring intensity of the luminescence reaction corresponding to the amount of ATP remaining after the reaction. The degree of autophosphorylation per 5 μg of protein kinase catalytic domain Pkb3 was 25%, per 10 μg – 43%, per 20 μg – 70%, thus revealing a linear dose-dependent relationship (Table 2). During the autophosphorylation reaction of the catalytic domains of protein kinases Pkb1, Pkb4, and Pkb6, the amount of ATP remaining after the reaction did not differ from that in the control sample.

The ability to autophosphorylate was found only in the catalytic domain of protein kinase Pkb3. In summary, our earlier data [4] and those obtained during this study demonstrate that only three of six STPKs are capable of autophosphorylation: Pkb2, Pkb3, and Pkb5.

The properties of mycobacterial STPKs were investigated most thoroughly. Strains of *M. tuberculosis* contain 11 serine/threonine protein kinases of eukaryotic type. All protein kinases from *M. tuberculosis* were cloned in *E. coli* (catalytic domain and/or full-size protein): an individualized approach was used to select the kind of vector plasmid for each protein kinase including optimized conditions for its expression, expression strain, developed isolation technique either under native or denaturing conditions, and optimized conditions for their renaturation. All protein kinases derived from *M. tuberculosis* were shown to undergo autophosphorylation [35-41].

In the current work we optimized conditions for culturing *E. coli* strains for further isolation of protein kinases and also developed methods for isolation of preparative amounts of proteins of *B. longum* six protein kinases' catalytic domains: a method for isolation of native proteins Pkb3 and Pkb4, a method for isolating protein Pkb5 in denatured conditions, and a method for isolating proteins

Table 2. Investigation of autophosphorylation of protein kinase Pkb3

Amount of STPK added to reaction mixture, μg	Amount of ATP remaining after kinase reaction, %	Autophosphorylation of STPK, %
5	75 ± 3	25 ± 3
10	57 ± 2	43 ± 2
20	30 ± 7	70 ± 7

Note: Average data from four independent repeats ± SD are presented.

Pkb1, Pkb2, and Pkb6 from inclusion bodies. In addition, a dialysis procedure for renaturation of proteins isolated from insoluble fraction was optimized.

The data of the present study on optimizing methods for isolation of preparative amounts of catalytic domains of six protein kinases of *B. longum* open new opportunities in investigating structure and functions of serine/threonine protein kinases from actinobacteria as well as creating their 3D crystal structure and looking for substrates.

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