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Cloning and expression of a recombinant immunogenic truncated BBK32 protein of *Borrelia afzelii*

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Abstract

Borrelia burgdorferi, the Lyme disease-causing spirochete, is often found associated with host connective tissue, where it interacts with components of the extracellular matrix, including fibronectin. BBK32 is a surface-expressed lipoprotein with fibronectin-binding ability of B*orrelia burgdorferi*. A fragment of the *bbk32* gene of *Borrelia afzelii* strain ACAI encoding the N-terminus of the protein including the fibronectin-binding domain (designated BS4 in this study) was cloned end expressed in *Echerichia coli* under the control of arabinose promoter as six histidine-tagged protein. Expression for the target protein showed that BS4 was accumulated both in soluble and insoluble forms. The molecular weight of the recombinant protein was estimated by SDS-PAGE to be 35 kDa including the six histidine tag. The expressed protein was purified by Ni^{2+} affinity chromatography under denaturing conditions. The purified BS4 recombinant protein was evaluated as an antigen in the serology of Lyme disease. Western blot analysis of Lyme disease patient sera revealed that the recombinant truncated BBK32 protein has specific antigenic properties. The availability of recombinant immunogenic BBK32 protein provides a new opportunity for biochemical analysis of the protein, structure-function studies, examination of its role in microbial pathogenesis, and assessment of its diagnostic and vaccinogenic potential.

Key words: antigenicity, *Borrelia afzelii*, recombinant protein BBK32.

Introduction

Lyme disease (Lyme borreliosis; LB), the most common arthropod-borne disease in Europe and North America, is caused by spirochete *Borrelia burgdorferi*. It is a multisystem disorder characterized by dermatologic, cardiac, neurologic, and arthritic manifestations (Steere 2001). A total of 488 cases of LB were recorded in Latvia in the year 2008, with incidence 21.49 per 100000 population. Lyme disease is spread to humans and other mammals via bites of infected *Ixodes* ticks (Burgdorfer 1982). In Latvia, two tick species, *Ixodes ricinus* and *Ixodes persulcatus*, transmit the disease. Our recent studies indicated that on average 25 % of ticks in Latvia are infected with Lyme disease spirochetes. All of three main genospecies of *B. burgdorferi* that are pathogenic in humans are prevalent in Latvia: *B. burgdorferi* sensu stricto, *Borrelia afzelii* (the most prevalent species in Europe) and *Borrelia garinii* (Ranka 2004). Very recently, the fourth pathogenic borrelia species *Borrelia spielmanii* was delineated in Europe (Richter 2006). Significant antigenic variation

between and within each genospecies has been described (Wang 1999), and among different strains of *B. garinii* this antigenic variability is more pronounced (Wilske 1996). Several studies have shown differences in immunoblot reactivity patterns depending on the strain, serotype, or species used as the antigen (Zoeller 1991; Mathiesen 1996; Hauser 1997; for review see Wilske 2007). The existence of such heterogeneity challenge many approaches of LB research, especially the development of diagnostic tests and vaccine.

In spite of numerous investigations of the nature of Lyme disease, knowledge of the pathogenicity and the virulence factors involved is still insufficient. The ability of *B. burgdorferi* sensu lato to encounter and persistently infect diverse vertebrate hosts frequently requires complex environmental sensing mechanisms involving coordinated expression of proteins essential to overcome host defence, in particular the innate and adaptive immune responses. Nevertheless, there is little information on the function of such proteins. Binding to host extracellular matrix molecules is one of the common strategies that bacterial pathogens employ for adhesion and invasion of host tissues. *B. burgdorferi* is capable of binding to a variety of host extracellular matrix molecules (Cabello 2007). Moreover, the interaction with host ligands mediated by spirochetal surface adhesins has been hypothesized to be critical for the pathogenic strategy (Guo 1995).

Lipoprotein BBK32 was identified as a typical fibronectin-binding adhesin of *B. burgdorferi* (Probert 1998). Recently it was shown that BBK32 induces the formation of fibronectin aggregates (Prabhakaran 2009) and is required for the initiation of microvascular interactions between borrelia and the host (Norman 2008). The BBK32 protein was also identified as an antigen that elicits an antibody response in infected mice as well as in Lyme disease patients, and it is a potential agent for serological test and vaccine development (Suk 1995; Fikrig 1997; Brown 2005; Lahdenne 2006; Scogman 2008). Recent studies showed that inactivation of the BBK32 gene in infectious strains of *B. burgdorferi* reduces spirochetal binding to fibronectin, as well as its infectivity in mice (Seshu 2006). However the mutants were shown to have no apparent defect in tick vectors (Li 2006).

The goal of the present study was to prepare a recombinant antigen using gene encoding BBK32 protein fragment in *B. afzelii* in order to obtain a molecule useful for pathogenicity, immunogenicity and virulence studies of Lyme disease spirochetes.

Materials and methods

Organisms and growth conditions

Cloning and expression of recombinant DNA was performed in *Escherichia coli* strain TOP10 (Invitrogen). Cells were grown in small-scale (10 mL) or medium-scale (50 mL) Luria-Bertani broth [10 g of NaCl, 5 g of yeast extract (Difco), 10 g of tryptone (Difco) per liter] supplemented with ampicillin (50 μg mL^{-1}, selective LB media), with aeration at 37 °C on a rotary shaker.

PCR amplification of BBK32 *gene and sequence analysis*

A PCR-based approach was used to amplify and sequence the *BBK32* gene from the ACAI isolate of *Borrelia afzelii* genomic DNA (kindly donated by S. Bergstrom, Umeå), as described elsewhere (Heikkila 2002). The PCR-amplified full-length *bbk32* gene was sequenced by standard technique using an ABI Prism 3100 Genetic Analyzer (Perkin-

Elmer, USA). The clone gene was conceptually translated and compared with sequences in the GenBank database using the BLAST program. Protein sequence analysis was carried out by using Software from the ExPASy Proteomics Server (Swiss Institute of Bioinformatics, http://www.expasy.ch/).

Construction and cloning of recombinant plasmid

Insert DNA encoding full length *BBK32* gene and *BBK* gene fragment were amplified by PCR from *B. afzelii* ACAI DNA.

The coding region corresponding to BBK32 (Ala2 - Tyr352) was amplified with 5' ccccatgggaaaaattaaaagtaaatg 3' and 5' ggctgcagtcaatggtgatggtgatggtggtaccaaacaccattctt 3', where the restriction sites are underlined. The coding region corresponding to BBK32 fragment (Ala2 - Lys215, designated as BS4) was amplified with 5' ccccatgggaaaaattaaaagtaaatg 3' and 5' ggctgcagtcaatggtgatggtgatggtgtttaacaccctctagata 3', where the restriction sites are underlined. Cloning primers were designed to be complementary to the BBK32 sequence of *B. afzelii* strain 600 (AF472528). *Nco*I and *Pst*I sites were arranged in the forward and reverse primers, respectively, and six histidine coding DNA fragments were arranged in the reverse primer. Apmlification of the target gene was confirmed by agarose gel electrophoresis. Restriction endonuclease digestion, ligation, transformation, isolation of plasmid DNA and other standart recombinant techniques were performed as described by Maniatis et al. (1982). Briefly, the amplified fragment was treated with restriction endonucleases, purificated from agarose gel with a DNA extraction kit (Fermentas) following manufacturer's instructions, ligated into modified pBAD/Thio-TOPO vector (Invitrogen), and transformed into TOP10 *E. coli* cells. The cells were plated onto Luria-Bertani agar plates containing 50 μg mL^{-1} ampicillin. Positive recombinant clones were selected by colony PCR. The *E.coli* cells from each positive colony were inoculated into 2 mL of LB medium containing 50 μg mL^{-1} ampicillin and grown overnight. The cells were harvested by centrifugation and plasmid DNA was isolated. Double-digestion of plasmid DNA by *Nco*I and *Pst*I was used for the identification of positive clones followed by restriction analysis in agarose gel. The recombinant clones were confirmed by DNA sequencing.

Small-scale expression of recombinant protein

Optimal growth and expression conditions for the protein of interest were established with small-scale cultures. *E. coli* TOP10 cells were transformed with expression plasmids. LB media containing 50 μg mL^{-1} ampicillin was inoculated with bacterial colonies and incubated overnight at 37 °C. The next day, selective LB media was inoculated with 1 % volume of the overnight culture and grown for 2 to 3 h at 37 °C or until OD_{600} reached 0.6. A few mL of cells were collected as an uninduced control before induction. Protein expression was induced with arabinose at different concentrations (0.2 - 0.00002 %, final concentrations), and induction was carried out at 37 °C for 2 or 4 h. Absorbance was measured, and an aliquot of cells from each tube corresponding to 2 units of OD was removed. Aliquots were centrifuged in a microcentrifuge (~ 2400 rpm) for 5 min, supernatant was removed, and pellets were frozen at –70 °C.

Expression of recombinant protein was confirmed by SDS polyacrylamide (10 %) gel electrophoresis (SDS-PAGE, as described by Laemmli 1970) and Western blot analysis (as described by Towbin et al. 1979) using an Amersham Hybond-C Extra

nitrocellulose membrane (GE Healthcare). SDS-PAGE separated proteins were visualized by Coomassie brilliant blue R250 staining or by silver staining as described elsewhere (Walker 1996). The Western blot procedure used PentaHis Antibody (Qiagen) and anti-mouse immunoglobulin G peroxidase conjugate (Sigma-Aldrich). The bound antibodies were detected using chemiluminiscent ELC Western Blotting Detection Reagents (GE Healthcare) following manufacturer's instruction or visualized by incubating the membrane with 3,3`-diaminobenzidine (Walker 1996). A Spectra™ Multicolor Broad Range Protein Ladder and Prestained Protein Molecular Weight Marker (Fermentas) were used as protein molecular weight markers during SDS-PAGE and Western blot analysis.

Preparation of soluble and insoluble protein fractions

Preparation of soluble/insoluble protein fractions was performed as described by Maniatis et al. (1982). The obtained samples were analyzed by SDS-PAGE and Western blot analysis as described above.

Expression and purification of the recombinant antigen

Cultures were grown in 50 mL of LB broth at 37 °C with aeration to an absorbance 0.5 - 0.6 at 550 nm. Arabinose was added to a 0.02 % final concentration, and induction was carried out at 37 °C for 4 h. The cells were harvested from 50 mL of culture by centrifugation at 6000 *g* at 4 °C; pellets were frozen at –70 °C for future use. Purification of recombinant protein was done under denaturing conditions using a Ni-NTA Spin Kit (Qiagen) following manufacturer's instructions with some modifications. Briefly, the cell pellet was resuspended in 10 mL Qiagen Urea buffer B containing DNAse I (5 μg mL^{-1}) and sonicated on ice. The insoluble materials were removed by centrifugation at 10 000 g for 20 min. The clarified supernatant was loaded onto pre-equilibrated Ni NTA Spin columns (Qiagen, 600 μL per column). After centrifugation, columns were washed twice, and absorbed proteins were eluted with 2 × 200 μL of Qiagen elution buffer E (pH 4.5) containing 8M urea. The obtained samples were analyzed by SDS-PAGE and Western blot analysis as described above.

Clinical samples

Five serum samples from patients with confirmed Lyme borreliosis were obtained from the Infectology Centre of Latvia. Samples were used in Western blotting (immunoblotting) analysis to determine reactivity to recombinant ACAI BBK32 proteins.

Additionally, sera from two borrelia noninfected humans (without LB in history and commercial Lyme test negative sample) were also used in these immunoblotting experiments. All human sera were diluted 1:1000 in PBS-Tween for immunoblotting.

Immunoblot analysis with clinical samples

Immunoblot analysis for the reaction between recombinant antigens and serum samples from Lyme disease patients was performed as following. The recombinant antigens separated by SDS-PAGE were electroblotted onto a nitrocellulose membrane. The membrane was cut onto slices, each slice contained a protein molecular weight marker (Fermentas) and recombinant antigen lines. Membrane slices were blocked by incubating in PBS containing 5 % skimmed milk and 0.1 % Tween 20 for 1 h at room temperature. After washing the membrane twice in PBST for 5 min, the membrane slice was incubated

with serum samples at 1:1000 dilution in PBS containing 0.1 % Tween 20 for 1 h at room temperature. After washing the membrane twice in PBST for 5 min, secondary probing was performed with protein A-peroxidase conjugate (Sigma) (1:1000 dilution in PBS containing 5 % skimmed milk and 0.1 % Tween 20) by incubating 1 h at room temperature. After washing twice in 0.1% Tween 20 PBS for 5 min, bands were detected with chemiluminiscent ELC Western Blotting Detection Reagents (GE Healthcare) following manufacturer's instruction or visualized by incubating the membrane with 3,3`-diaminobenzidine (Walker 1996).

Results

Sequence analysis

Sequence analysis of the amplified *bbk32* gene revealed 99 to 100 % homology with the *B. afzelii bbk32* genes from different isolates (*B. afzelii* strain 1082, A91, PKo, 600, 570, ACAI; data not shown). The deduced amino acid sequences of the BBK32 protein contained 352 residues. Secondary structure prediction analysis indicated that the *B. afzelii* ACAI BBK32 protein consisted mostly of alpha-helical regions with a few beta sheets. A signal peptide region (amino acids 1 - 20) and transmembrane region (amino acids 9 - 24) were also predicted. The greater part of the mature portion of the BBK32 protein was hydrophilic. The calculated molecular size of the mature BBK32 protein (without histidine tag) was 40.7 kDa. The obtained information was used for design of a truncated BBK32 variant. The sequence was selected from areas of predicted hydrophilicity including the N-terminal and mid-portion of the mature sequence (amino acids 2 - 215). The fibronectin-binding domain of BBK32 (amino acids 131 - 162; Probert 2001) was also included.

Cloning of the bbk32 *gene*

The DNA-coding region for the mature BBK32 (nucleotides 4 - 1059) and for the truncated BBK32 (nucleotides 4 - 645) of *B. afzelii* strain ACAI were produced by PCR and cloned into a modified bacterial expression vector pBAD/Thio-TOPO (Fig. 1).

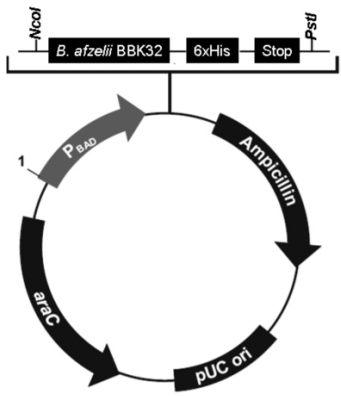

Fig. 1. Expression vector for *B. afzelii* BBK32. *B. afzelii* ACAI BBK32 coding sequence was inserted into modified pBAD/Thio-TOPO vector containing the ampicillin resistance marker. Expression of the recombinant protein was induced by arabinose.

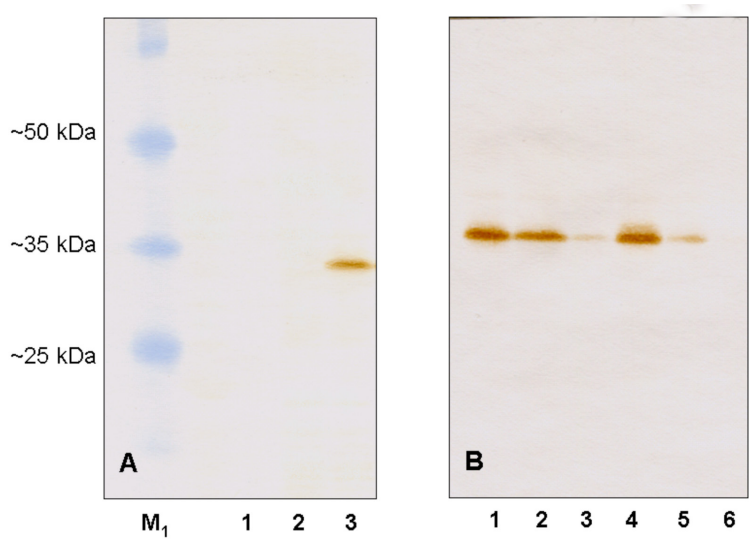

Fig. 2. Expression of recombinant protein BBK32 of *B. afzelii* in *E. coli*. Proteins from *E. coli* lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane and proteins of interest were detected by Western blotting using antibodies against His tag. Fragment of *bbk32* gene from *B. afzelii* ACAI was cloned into the modified pBAD/Thio-TOPO vector and expressed as a fusion protein with a six-histidine tag at its C-terminus (A, panel 3). The recombinant protein expression was not detected before the induction (A, panel 2). Expression of full-length recombinant *bbk32* gene was not detected (A, panel 1). Panel M_1 – protein molecular weight marker. The effect of different arabinose concentration (final concentration is shown) in the expression of recombinant truncated BBK32 protein at different expression time points (B). Panel 1 – 0.2 % Ara, 2 h; panel 2 – 0.002 % Ara, 2 h; panel 3 – 0.00002 % Ara, 2 h; panel 4 – 0.2 % Ara, 4 h; panel 5 – 0.002 % Ara, 4 h; panel 6 – 0.00002 % Ara, 4 h.

To verify the primary selection, each single colony was cultured and plasmid DNA was isolated. Plasmid DNA digestion by *Nco*I and *Pst*I restriction enzymes and DNA sequencing confirmed successful insertion of the *bbk32* gene and *bbk32* gene fragment into the modified pBAD/Thio-TOPO vector. Total length of the inserted DNA fragment with the six histidine tag was 1074 nucleotides encoding 357 amino acids and stop codon (designated BBK32ACAI), and 669 nucleotides encoding 222 amino acids and stop codon (designated BS4), respectively. The C-terminal histidine tag was used to affinity-purify the fusion protein by chelated Ni^{2+} resin. The obtained constructs were transformed into *E. coli* strain TOP10 for protein expression.

Expression of the recombinant antigen

The recombinant antigen candidates were expressed in *E. coli* strain TOP10, expression was proved by SDS-PAGE and western blot analysis with PentaHis antibodies (Qiagen) and Anti-mouse HRP antibodies (Sigma). The antigen BS4 was identified as a protein with molecular mass of approximately 35 kDa (Fig. 2). Bacterial expression was optimized by analysis of protein expression on different amounts of arabinose (Fig. 2B). *E. coli* containing recombinant vector expressed BS4 only after arabinose induction, but no significant increase of recombinant protein production was observed at different time

points (Fig. 2). Analysis of soluble and insoluble protein fractions showed that BS4 was expressed both in soluble and insoluble forms (data not shown).

Negligible amounts of antigen BBK32ACAI were detected in SDS-PAGE and western blot analysis, indicating that the expression of full-length BBK32 protein is problematic under the conditions used (Fig. 2).

Purification of recombinant antigen

To establish whether the recombinant truncated BBK32 protein could be purified via the C-terminal histidine tag, the cleared lysates were subjected to affinity chromatography on Ni-NTA Spin columns under denaturing conditions. The flow-through, wash and eluted fractions were collected and analyzed by SDS-PAGE and/or Western blotting (Fig. 3). The results showed that BS4 could be purified by this method; however, some modifications of procedure will be necessary in future. The results show that the capacity of used spin column was not enough to bind all of the expressed protein, because it was still detectable in significant amounts in the flow-through fraction. Western blot analysis showed that some extra bands of smaller size appeared in elution fractions in addition to the protein of interest indicating possible protein degradation during the purification procedure. A few other contaminants were copurified with the protein of interest under the conditions used, observed in the affinity purified fractions on SDS-PAGE (Fig. 3). Nevertheless, the quality of the obtained purified BS4 protein was good enough to perform further work.

Immunoreactivity of the recombinant antigens with patient serum

Five Lyme disease patient sera and two negative control human sera were used in immunoblott analysis with the recombinant BS4 antigen. In all reactions, the BS4

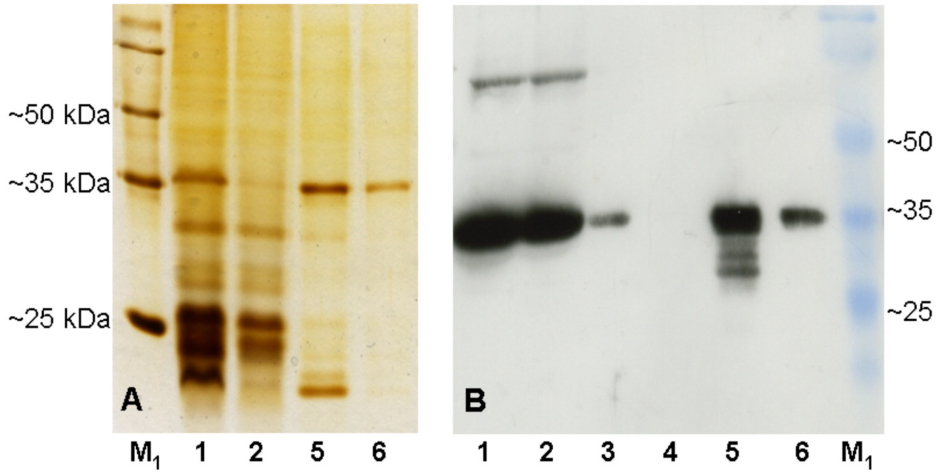

Fig. 3. Purification of recombinant truncated BBK32 protein. Protein of interest (BS4) was expressed in *E. coli* and purified by Ni^{2+} affinity chromatography. Proteins were separated by SDS-PAGE and detected by silver staining (A) or transferred to a nitrocellulose membrane for Western blotting with antibodies against His tag (B, chemiluminiscent detection). Panel 1 – *E. coli* crude cell lysate; panel 2 – *E. coli* cell lysate, flow-through; panel 3 – first wash fraction; panel 4 – second wash fraction; panel 5 – elution fraction 1; panel 6 – elution fraction 2; panel M_1 – protein molecular weight marker.

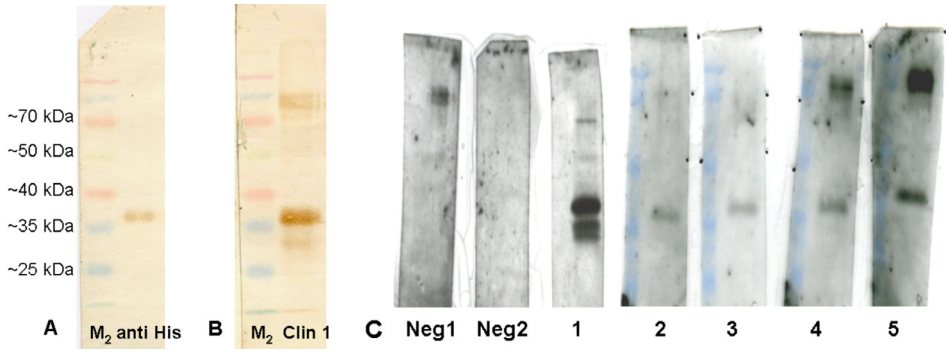

Fig. 4. Western Blot analysis of Lyme disease clinical samples with BBK32 protein. The truncated BBK32 protein of *B. afzelii* ACAI (BS4) was expressed in *E. coli* as a recombinant protein with six His tag. The protein was purified by Ni^{2+} affinity chromatography and the elution fraction 1 was used as an antigen in Western blot analysis. Probing with antibodies against His tag (A, detection by 3,3`-diaminobenzidine); probing with Lyme disease serum sample from patient No. 1 (B, detection by 3,3`-diaminobenzidine). Panel M_2 – protein molecular weight marker. Probing with different clinical samples (C, chemiluminiscent detection), Neg1 and Neg2 – Lyme disease negative serum samples; 1 - 5, Lyme disease positive serum samples.

elution fraction 1 was used. The results demonstrated that recombinant BS4 antigen had significant reactivity with Lyme disease patient sera (Fig. 4). Some differences in the immunoreactivities of the serum samples against BS4 protein were observed.

No positive reaction was observed between recombinant antigen and Lyme disease negative patients sera samples (Fig. 4).

Discussion

Since pathogenic mechanisms of Lyme borreliosis is still mostly unclear, detailed studies of the biological activity of borrelia specific proteins with antigenic nature are essential for further studies. A better understanding of the nature of predominant antigens of *Borrelia burgdorferi* will be useful in the attempt to detect factors involved in virulence and the study of pathogenicity, and also for serological differentiation of *Borrelia* species and for development of a vaccine. In search for these appropriate antigens with specificity and sensitivity, a large number of research papers have been published. These studies have helped to determine some spirochetal virulence factors (for review see Steere 2005), but Lyme disease pathogenesis is still poorly understood.

In this study we have cloned and expressed in *E. coli* the gene fragment of an immunogenic protein BBK32 from *B. afzelii* ACAI (designated BS4 in this study). Expression analysis showed that the maximal recombinant protein amount was produced after induction with 0.2 % arabinose (final concentration). However, the protein amount did not differ at time points 2 h and 4 h after induction (Fig. 2B). Analysis of soluble and insoluble protein fractions displayed that the protein of interest exists both in soluble and insoluble forms. In this study, to obtain maximal protein yields we purified BS4 under denaturing conditions. Western blot analysis of the obtained fractions showed that under these conditions certain amount of proteolysis occured shown by the appearance of some

new bands of proteins with smaller molecular weight that reacted with anti-His antibodies.

In this study we tested the antigenicity of truncated BBK32 protein of *B. afzelii* by using Lyme disease patient sera in Western blot analysis. We focused on *B. afzelii* proteins, because it is the most prevalent species of Lyme disease borrelias in Europe and also in Latvia (Steere 2001; Ranka 2004). We have shown that sera from Lyme disease patients recognize this recombinant protein. This finding is in accordance with the data of Lahdenne et al. (2003; 2006). However, these authors used full-length BBK32 protein and shorter mid-portion fragments of BBK32 (amino acid range 130-220) in ELISA studies. Our findings enhance the evidence that the BBK32 proteins are promising serodiagnostic antigens for the detection of LB. Variant BBK32 proteins may be used either in parallel or in combination in an immunoassay for LB to cover all the relevant borrelial species, whose prevalences differ regionally in Europe. In future studies, it will be interesting to identify possible antigenic epitopes of *B. burgdorferi* BBK32 proteins. There is some evidence that variant BBK32 proteins may have both individual and common antigenic epitopes and that epitope specificity varies in early and late LB (Lahdenne 2006).

In conclusion, we have been able to clone and express the N-terminal part of BBK32 protein of *B. afzelii*. This protein showed clear antigenic properties in Western blot analysis with Lyme disease patients' sera, which makes it useful for further studies of pathogenicity and for diagnostic applications of Lyme borreliosis.

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