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Prokaryotic Expression Purification and Protein Activity Detection of Histidinekinase VicK from *Streptococcus mutans*

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To express and purify the histidinekinase VicK from *Streptococcus mutans* and to detect its protein activity. The VicK gene was synthesized and constructed into the prokaryotic expression vector pET28a by DNA recombination techniques. After the construction was confirmed correctly by enzymatic digestion and sequence analysis, the products of expression which was induced by IPTG were analyzed by SDS-PAGE. The activity of purified target protein was detected by Kinase-Glo® Luminescent Kinase Assays. Vick cDNA was cloned into pET28a plasmid successfully. Using 0.3mmol/L IPTG at 18! for 15 h, the soluble target protein was expressed efficiently ,and was purified by Ni affinity column .The target protein of 2mg/ml has does-dependent kinase activity of hydrolyzing ATP in vitro. The successful expression and purification of vick protein which has does-dependent kinase activity provided basis for further research of inhibitors screening targeting Vick protein of *streptococcus mutans*.

Key words: *Streptococcus mutans,* Histidinekinase, Prokaryotic expression, Purification, Enzymatic activity.

Streptococcus mutans, a major dental caries pathogenÿadhere to the surface of tooth with other bacteria to form the biofilm , and its cariogenic effect is often affected by the environment with the quorum sensing system which is the main signal transduction system. As a significant part of sense of changing environment and control of gene expression , the Two-component System which is one of the important quorum sensing system is necessary for bacterial adaptation, survival, and virulence[010].The changes in the external environment which are converted into signal molecules by phosphorylating with histidinekinase of TCS are transferred to inside to start a series of gene transcriptions, expressions and modifications of expressed product. Among 13 TCSs identified in *S. mutans*[020], VicRK as a ETCS what is essential for bacterial survival and highly conservative affects some important physiological processes of bacteria such as biofilm formation, gene expression, acid tolerance and response of oxidative stress,etc[04-70]. Because the TCS has not yet been found in mammals (including humans),it is possible to screen out the inhibitor targeting TCS that can reduce the pathogenicity of *S. mutans* to prevent cavities .In this study, vick protein which has does-dependent kinase activity were expressed and purified successfully by using template DNA from *S. mutans*, this provided basis for further research of inhibitors screening targeting it.

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MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial lyophilized powder of *S. mutans* UA159 provided by the State Key

Laboratory of Oral Diseases of Sichuan university, the *E.coli* BL21 DE3 and pET28a plasmid provided by State Key Laboratory of Clinical Laboratory Diagnostics of Chongqing medical university.

Main reagents and instruments

The primer STAR DNA polymerase, restriction enzyme EcoR'! and Xho'! and DL2000 DNA marker were bought from TaKaRa Biotechnology (Dalian);the Anti-His Tag , Ni affinity chromatography column bought from Merk company ; the Kinase-Glo® Luminescent Kinase Assays, ATP and T4 DNA Ligase bought from Promega company.

Design and synthesis of primer

A pair of primer has been designed using the vick intracellular gene from *streptococcus mutans* according to the GenBank (ID:1028760) as a template. The following are the primer sequences: vick-F,5'-GCGCGAATTC ATGGTTAAACAGTTAAATGCT-3' (the under lined part is restriction enzyme site EcoR'!); Vick-B: 5'-ATCCTCGAGTCATG ATTCGTCTTCAT CTTCT-3' (the underlined part is restriction enzyme site Xho'!)

Amplification of the target gene

The genomic *DNA* of *S.mutans* UA159 cells which were cultured in Brain Heart Infusion Broth (BHI) media until logarithmic phase were extracted by DNA extraction kit. The Vick gene was amplified from 2 ìl *S.mutans* UA159 genomic *DNA,* the reaction conditions were as follows, pre-denaturalise at 98! for 5min; denaturalize at 98 for 10s; anneal at 57! for 45s; elongate at 72! for 80s; followed by 35 cycles of 2 to 4 steps; elongate again at 72! for 10min. The PCR-amplified fragment was identified by 1ÿagarose gel electrophoresis and purified by purification kit.

Construction of recombinant plasmids

The purified PCR-amplified fragments was digested with EcoR'!and Xho'!and ligased to vector pET28a using T4 DNA ligase at 16! for 16h.The resulting plasmid , pET28a-VicK ,was introduced into E.coli strain BL21(DE3).The transformed cells were first cultured overnight in Luria Broth (LB) solid media with 50mgÿml kanamycin at 37 !,and selected for resistance to kanamycin .The *E. coli* strain BL21 cells containing pET28a-VicK were grown in LB liquid media with 50mgÿml kanamycin at 37°C with shaking. The plasmids extracted from the bacteria before were confirmed correctly by PCR , enzymatic digestion and sequence analysis.

Expression and purification of target protein

The positive clone bacteria were diluted 100-fold in 3ml fresh Luria Broth (LB) liquid media with 50mgÿml kanamycin and cultured at 37 ! with shaking for approximately 4h. When optical density at 600 nm (OD600) reached to 0.6,the cells were induced with 0.0100.0200.0500.100.2 and 0.3mmol/L IPTG respectively at 18! and 37!.The IPTG was absent for blank control group. The cells induced for 10015 and 20h respectively were harvested and analyzed which was the best condition for reduce by SDS-PAGE. The 200ml bacterial media which were reduced using the condition screened out were harvested and disrupted by sonication for 10min. The cell debris and supernatant were used to analyze expression of target protein by SDS-PAGE. The sample which had plenty of target protein in supernatant was concentrated at 16710*g for 30min , and the cell debris was removed. The soluble lysis fraction was loaded onto a nickel-chelation column equilibrated in Binding Buffer ÿ20mmolÿL Trisÿ 500mmolÿL NaClÿpH7.9 ÿand the target protein was then eluted by a liner gradient of 20 to 500 mmol/L imidazole in Binding Buffer. The protein content in different concentration of imidazole was analyzed by SDS-PAGE. The VicK fractions were collected and concentrated by ultrafiltration and then analysed the purity by SDS-PAGE.

Activity identification of target protein

The Kinase-Glo® Luminescent Kinase Assays was used to determine the kinase activity of target protein Vick. A different quantity of VicK protein (2,4,6,8,10ìg) were diluted to 45ml with reaction buffer(40mmolÿL Tris-HclÿpH7.5 ÿÿ20mmolÿL MgCl2ÿ0.1mgÿml BSA) respectively and added 5ìL ATP causing the each final concentration was 5ìmolÿL. After reaction at 37! for 10min, 50ìL Kinase-Glo® reagent was added, and then reacted again at 37! for 10min. The OD value was measured in 560nm project laser.

RESULTS

Identification of recombinant plasmid

The image of o1ÿagarose gel electrophoresis revealed that there were target

fragments (1100bp) in the products amplified by PCR using *S.mutants* UA159 and digested by enzyme using recombinant plasmid.Fig.2. The sequencing result was consistent well with information from GenBank.

Identification of the purified protein

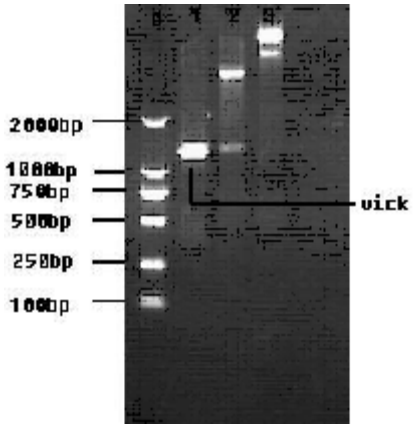
The expression products of induced bacteria containing plasmid pET28a-VicK as a bright band about 51000Dolton by SDS - PAGE analysis, this was accorded with theoretical value, and it enhanced most significantly after 15h of inducing at 18! with 0.3mmol/L IPTG . The SDS-PAGE analyzed the purity of purified recombinant protein VicK was above 90 % (Fig.2), and its concentration was 2mg/ml.

Activity identification of the target protein VicK

Through activity analysis, the concentration of ATP in the range of 1.25~10ìmol/L had a good linear relation with fluorescence intensity(2^2"0.9753) (Fig3) so that it can be calculated via the value of fluorescence intensity. The data from kinase activity detection of VicK was reliable when the initial and final reaction concentrations of ATP in this range. After the same amount of ATP were added to reaction system, the fluorescence intensity in the reaction system decreased with increasing quantity of VicK protein (Fig.4). This illustrated the target protein had does-dependent kinase activity of hydrolyzing ATP in vitro.

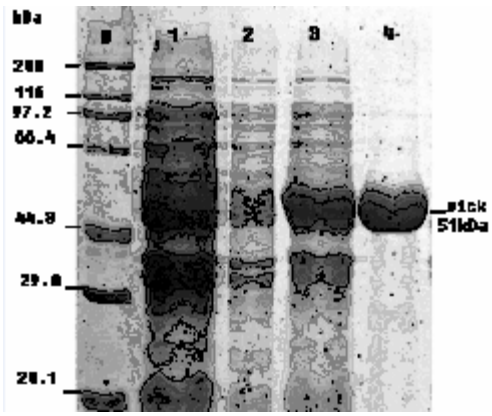
DISCUSSION

The two-component system which consists of the sensor protein (histidine protein kinase, HK) and the response-regulator protein(RR) is main signal transduction system in bacteria.



0 DNAmarker DL2000 ; 1 The PCR products; 2 The pET28a-VicK digested with EcoR'!ÿXho'!; 3 The recombinant plasmid pET28a-VicK

Fig 1. Identification of recombinant plasmid pET28a-VicK



0 Protein marker; 1 pET28a; 2 Non-induced BL21 with plasmid pET28a-VicK; 3 Induced BL21 with plasmid pET28a-VicK; 4 urified protein of VicK(approximately

Fig 2. SDS-PAGE of pET28a-VicK protein expressed in BL21

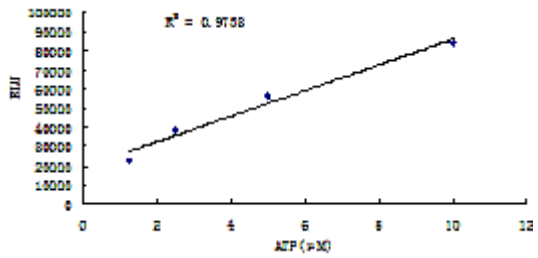


Fig 3. Luminescent output correlates with amount of ATP

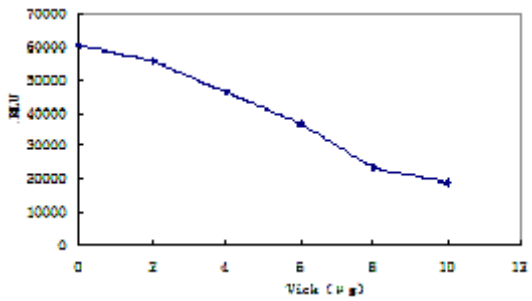


Fig 4. Luminescent output is inversely correlated with VicK' protein activity in vitro

Through phosphorylation reactions, extracellular signals sensed by the sensor protein (HK) are transferred to the cognate response regulator protein (RR), which mediates gene expression. In *Streptococcus mutans,* essential physiological processes may be modulated via the two-component regulatory system VicRK. As one part of the VicRK , the deletion of VicK can cause decreased living or even death,so drugs acted on VicK can inhibit the growth of bacteria or kill germs. The getting of does-dependent kinase activity of hydrolyzing ATP in vitro is the key to screening of effective inhibitor. SMART database ÿhttp://smart.embl-heidelberg.de/ ÿ a n a l y s i s were performed to know the *S. mutans* VicK. It is a transmembrane protein contains four main conserved domains (PAS, PAC,HisKA, HATPase_c),and the HATPase_c domain acts on ATP binding. It is difficult to have does-dependent kinase activity of hydrolyzing ATP in vitro for whole transmembrane protein, so we chosen to express the endocellular part of VicK including HATPase_c domain^{080}. Expressed protein takes the form of inclusion body and solubleness**.** Inclusion body protein doesn't have biological activity in general for its false spatial location, though it is made up of protein. We optimized the condition for a better expression of VicK protein in order to avoid being disturbed of the kinase activity of induced protein by plenty of inclusion body protein, and then obtained high - expressed soluble protein VicK induced for 15h at 18! using 0.3mmol|L IPTG After repeated optimization about induce time, temperature, concentration of IPTG, etc. The Kinase-Glo® Luminescent Kinase Assay measure kinase activity by quantitating the amount of ATP remaining in solution following a kinase reaction. In this study, the conserved kinase domain needed ATP to be hydrolyzed to finish autophosphorylation caused decreasing of the amount of ATP remaining in reaction system so that the OD value was reduce, this indicated induced protein had kinase activity. The successful expression and purification of VicK protein which has dose-dependent kinase activity provided basis for further research of inhibitors screening targeting Vick protein of *streptococcus mutans*.

ACKNOWLEDGMENTS

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