Protein Expression and Purification 64 (2009) 162–166

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Cloning, expression and purification of DNA-binding protein Mvo10b from *Methanococcus voltae*

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ARTICLE INFO

Article history:
Received 8 September 2008
and in revised form 7 November 2008
Available online 17 November 2008

Keywords:
Mvo10b
Methanococcus voltae
DNA-binding protein
Purification
Nucleic acid contamination

ABSTRACT

Mvo10b from the mesophilic archaeon *Methanococcus voltae* is a member of the Sac10b family which may play an important role in the organization and accessibility of genetic information in Archaea. Since Mvo10b is a DNA-binding protein as the other member in the Sac10b family, to obtain a recombinant Mvo10b requires an efficient and inexpensive expression and purification system for producing the protein free of nucleic acid contamination. Previously, the hyperthermophilic archaeal Ssh10b of the Sac10b family was successfully purified. However, the protocol adopted to purify Ssh10b is not appropriate for purifying the mesophilic Mvo10b. This study describes the successful expression and purification of the recombinant Mvo10b. The expression of recombinant Mvo10b was carried out in *Escherichia coli*, and the target protein was expressed in the soluble form. The protein was purified by polyethyleneimine (PEI) precipitation followed by nickel ion metal affinity chromatography. The purity of Mvo10b was checked to insure being free of nucleic acid contamination. The final protein yield is about 30 mg/l of LB culture. The ensemble of NMR and far-UV CD data shows that the purified Mvo10b has abundant regular secondary structures and is correctly folded, which may have similar 3D structure as its hyperthermophilic counterpart [P62A]Ssh10b. The developed protocol has potential application in the production of the other thermophilic and mesophilic proteins in the Sac10b family.

Three domains of organisms, the Eucarya and the prokaryotic Archaea and Bacteria, have been classified mainly based on rRNA sequence comparisons [1,2]. Archaea and Bacteria share the same basic mechanism of translation, which differs from that used by the Eucarya. The archaeal DNA replication and transcription pathways are very similar to those in Eucarya but quite different from the equivalent bacterial processes [3]. In the Eucarya, the chromosomal DNA is compacted by the interactions with proteins, such as histones, forming a higher-order structure of chromatin. Bacterial histone-like proteins (HU, IHF, H-NS, etc.) interact with DNA in a variety of ways [4]. The archaeal chromatin proteins are different from those found in Bacteria. A set of small and basic DNA-binding proteins has been identified in Euryarchaea [5]. Recently, a Cren7 family of chromatin proteins has been identified in Crenarchaea, and it was suggest that the majority of Crenarchaea share a common strategy in chromatin organization [6].

Sac10b from the kingdom Crenarchaeota is the second most abundant protein in the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* [7]. It has been shown that Sac10b binds cooperatively to DNA and forms different protein–DNA complexes depending on protein concentration [8]. A gene denoted Ssh10b encoding a protein homologous to Sac10b has been purified from the hyperthermophilic archaeon *Sulfolobus shibatae*, which has at least one homologue in each of all archaeal genomes, but not in either Bacteria or Eucarya [9]. These homologues form the Sac10b family [9,10]. Proteins of the Sac10b family are highly conserved among thermophilic and hyperthermophilic Archaea, and homologous sequences have also been identified in eucaryal proteins from higher plants, protists, and vertebrates.

Mvo10b from the mesophilic archaeon *Methanococcus voltae* having an optimum growth temperature of 35 °C is a member of the Sac10b family. Although the members from hyperthermophilic Archaea in Sac10b family have been studied extensively, little is known about mesophilic members in this family. The aims of the work described here were expression and purification of Mvo10b for further structure–function study by various biochemistry and biophysics methods. The reported procedure for purifying hyperthermophilic archaeal Ssh10b [11] is not appropriate for Mvo10b since it cannot effectively remove nucleic acid contaminants. The present work reports a new approach to express and purify highly pure Mvo10b, which can be used for the production of the other thermophilic and mesophilic proteins in the Sac10b family.

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doi:10.1016/j.pep.2008.11.003

Materials and methods

Chemicals and solutions

A 5% polyethyleneimine (PEI)[1] stock solution was prepared for removing the nucleic acid contaminants from the target protein. For preparing this solution, 100 ml 50% (w/v) polyethyleneimine (Sigma) was dissolved in deionized water with a final volume of 1000 ml. The pH of the solution was adjusted by concentrated HCl to a reading of 7.9.

Five different buffers were prepared for this study. They are buffer A, 50 mM Tris–HCl buffer (pH 8.0) containing 1 M NaCl; Buffer B, 50 mM sodium phosphate buffer (pH 6.0) containing 500 mM NaCl; Buffer C, 50 mM sodium phosphate buffer (pH 6.0) containing 2 M NaCl and 50 mM imidazole; Buffer D, 50 mM sodium phosphate buffer (pH 6.0) containing 500 mM NaCl and 500 mM imidazole; Buffer E, 50 mM potassium phosphate buffer (pH 6.0) containing 100 mM KCl.

Strains and plasmid construction

A DNA fragment encoding the gene Mvo10b was amplified by polymerase chain reaction (PCR) using a sense primer, 5′-GGAA TTCCATATGGAAAATTC-3′, and an anti-sense primer, 5′-CCGCTCG AGTTCTTTTGAAATTG-3′. The primers were introduced to an NdeI site (sense) and an XhoI site (anti-sense), respectively. The amplification was performed using 2×pfu PCR MasterMix (TIANGEN BIOTECH, Beijing, China) on an Eppendorf PCR instrument. The PCR products were purified by 1% agarose gel electrophoresis and double digested and ligated into the expression vector pET22b(+) (Novagen), resulting in a pET22b-Mvo10b plasmid with the sequence encoding the gene Mvo10b. The constructed plasmid was transformed into competent *Escherichia coli* TOP10 cells and was identified by DNA sequencing.

Bacterial expression of recombinant Mvo10b

The recombinant plasmid pET22b-Mvo10b was transformed into *E. coli* expression strain BL21(DE3) cells. One colony was picked up and was grown in 30 ml LB rich medium containing 100 mg/l ampicillin. After 8 h, 10 ml of the BL21(DE3) cells were introduced into 1 l of LB medium containing 100 mg/l ampicillin. Bacteria were grown at 37 °C until an OD_{600} of 0.8 was reached. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression of the protein for 4 h at 37 °C. The cells were harvested by centrifugation at 4800g for 30 min.

Purification of recombinant Mvo10b

The cell pellet recovered from 1 l culture was resuspended in 30 ml of buffer A and lysed by sonication. After centrifugation at 30,700g for 30 min, the supernatant containing proteins with nucleic acid contamination was poured into a beaker in an ice bath. Then, the proteins were separated by precipitating the nucleic acids using 5% PEI. Five percent PEI stock solution was added progressively into the extract with gently stirring until the final concentration of PEI was 0.8%. During this process, the nucleic acids were precipitated. The precipitation was removed by centrifugation at 17,300g for 10 min, leaving the proteins in the supernatant. Crystalline ammonium sulfate was added slowly into the supernatant up to 474 g/l (70% saturation) with gently stirring for 30–45 min, resulting in the protein precipitation. After centrifugation at 30,700g for 30 min, the pellet was repeatedly washed by 70% saturated $(NH_4)SO_4$ solution to remove the excess free PEI. The pellet was then resuspended with 30 ml buffer B.

The protein was then purified by a nickel-column affinity chromatography followed by a gel filtration. The nickel-column with Chelating Sepharose™ Fast Flow media (Amersham Pharmacia Biotech, Sweden) was first equilibrated with 0.2 M nickel chloride, followed by de-ionized water to remove excess nickel ions from the column, and then equilibrated with the buffer B. The protein sample was applied onto the column which was initially washed with five column volumes of the buffer B followed with buffer C. The protein was eluted with buffer D and then concentrated to 2 ml by Amicon Ultra-15 Centrifugal Filters (Millipore, USA). The collected protein solution was then applied to a Superdex G75 (Amersham Pharmacia Biotech, Sweden) column pre-equilibrated with buffer E on an ÄKTA purifier-10 system. The fractions eluting from the Superdex G75 column was collected and then concentrated by ultrafiltration. The final protein concentration was about 5 mg/ml, and the purified protein was stored at −20 °C. The purity of protein was analyzed by 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and by a ratio of $A_{280}/A_{260} \geqslant 1.7$ to insure being free of nucleic acid contamination [12,13].

Far-UV circular dichroism (CD) measurements

The far-UV CD measurements were performed with Mvo10b on a PiStar spectropolarimeter (Applied Photophysics, Surrey, UK) at 25 °C. The protein sample was 0.5 mg/ml in buffer E. Measurements were carried out with a 1 mm path-length cuvette over the wavelength range from 195 to 260 nm at 1 nm bandwidth. Multiple scans were averaged for the collected spectrum. The spectrum was processed by subtracting the buffer spectrum. The ellipticity of the far-UV CD spectrum was analyzed using the software DICROPROT (download from http://pbil.ibcp.fr/htm/index.php?page=pbil_ibcp_Software.html) [14].

NMR spectroscopy

For NMR experiments, the uniformly ^{15}N-labeled Mvo10b was obtained by growing the recombinant strain in M9-minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source. The labeled proteins were purified as described above. The purity of proteins was checked by SDS–PAGE to ensure a single band. Samples for NMR experiments were 1.0 mM ^{15}N-labelled Mvo10b in 90% H_2O/10% D_2O containing 50 mM potassium phosphate buffer (pH 6.0), 100 mM KCl, 0.01% sodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS), and 0.01 mM NaN_3.

All NMR experiments were carried out at 310 K on a Bruker DMX 600 MHz spectrometer equipped with a *z*-gradient triple-resonance cryo-probe. The 2D ^1H–^{15}N HSQC and 2D ^1H–^1H plane of 3D ^1H–^{15}N NOESY-HSQC spectra were recorded for ^{15}N-labeled Mvo10b. All NMR data were processed and analyzed with FELIX98 software (Accelrys Inc.). The data points in each indirect dimension were usually doubled by linear prediction before zero filling to the appropriate size. A 90° shifted square sine bell apodization was used for all dimensions before Fourier transformation. ^1H chemical shifts were referenced to internal DSS. ^{15}N chemical shifts were referenced indirectly [15].

Results and discussion

Expression and purification of Mvo10b free of nucleic acid contaminant

Mvo10b was overexpressed in *E. coli* after induction by IPTG (Fig. 1). The induction time was optimized to 4 h for obtaining

[1] *Abbreviations used:* IPTG, isopropyl-β-D-thiogalactopyranoside; DSS, sodium-2,2-dimethyl-2-silapentane-5-sulfonate; PEI, polyethyleneimine; PCR, polymerase chain reaction; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

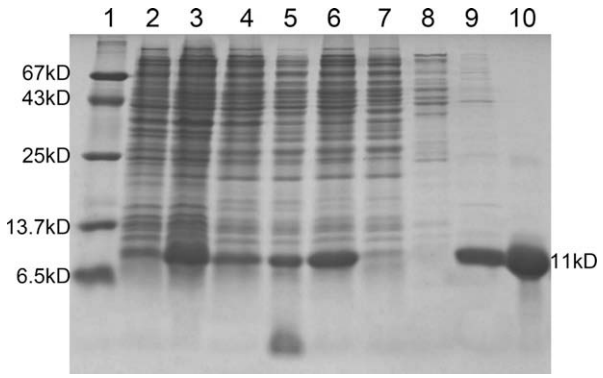

Fig. 1. SDS–PAGE analysis of Mvo10b expression and purification in *E. coli*. Lane 1, molecular weight marker with molecular weights indicated; lane 2, cell lysate before IPTG induction; lane 3, cell lysate after IPTG induction for 4 h; lane 4, supernatant after sonication; lane 5, PEI treated supernatant product; lane 6, sample before Ni^{2+}-affinity chromatograph; lane 7, flow-through fraction from Ni^{2+}-affinity chromatograph; lane 8, column wash with the buffer containing 50 mM imidazole; lane 9, elution in 500 mM imidazole from Ni^{2+}-affinity column; lane 10, purified Mvo10b after Superdex G75 gel filtration.

the highest yield and soluble fraction. The expression levels in *E. coli* strains BL21(DE3), BL21(DE3)pLysS, Rossetta(DE3), and Rossetta(DE3)pLysS are similar (data not shown). Mvo10b was mainly soluble in the supernatant after lysing (Fig. 1, lane 4).

The protocol adopted to purify hyperthermophilic Ssh10b is not appropriate for purification of mesophilic Mvo10b. In the purification of Ssh10b, the supernatant after overexpression of the protein was heated for 20 min at 80 °C, precipitating the nucleic acid contaminants and most of unwanted *E. coli* proteins. After centrifugation, the supernatant was applied to a Resource-S column followed by a gel filtration [11]. Using these procedures, the successful purification of Ssh10b was achieved. However, the heat treatment used for purifying hyperthermophilic Ssh10b cannot be applied to purify mesophilic Mvo10b. On the other hand, using only the cation ion-exchange chromatography followed by a gel filtration can provide one band in SDS–PAGE. However, Mvo10b obtained using this two-step procedure was contaminated by a great quantity of nucleic acids when the effluent after Superdex G75 chromatography was monitored by A_{280}/A_{260} absorbance ratios [16]. The observed A_{260} was much greater than A_{280} having an A_{280}/A_{260} absorbance ratio of 0.42 (Fig. 2), indicating the large fraction of nucleic acids which

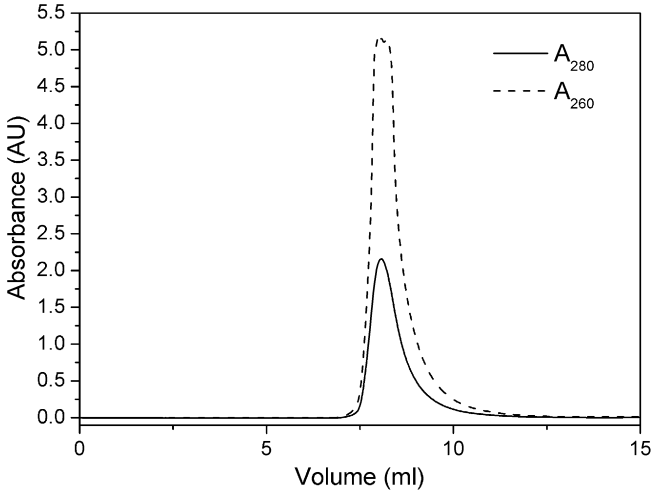

Fig. 2. Superdex G75 (column volume 24 ml) chromatography of Mvo10b purified by a protocol similar to that for purification of Ssh10b. The effluent of each sample was monitored by absorbance A_{280} (solid line) and A_{260} (dashed line) simultaneously.

appeared as a smear on 1% agarose gel electrophoresis of the protein.

To develop a new method for obtaining purified Mvo10b free of the nucleic acid contamination, approaches such as addition of protamine sulfate, streptomycin, magnesium chloride, spermine, and the appropriate nucleases [17,18] were inspected. Nevertheless, they were not appropriate for removing nucleic acids from Mvo10b. Moreover, the methods of treatment with Benzonase (Benzonase®, Merck) and polyethyleneimine (PEI) [19] and the method using the positively charged anion-exchange resins [20] were tested in the purification of Mvo10b. However, the method of treatment with Benzonase costs a lot and the interested protein would always co-precipitate with nucleic acids. On the other hand, the efficiency of ion-exchange chromatography using CM-cellulose was too low to remove nucleic acids, and most of the proteins flowed through the column. Probably, the tight binding of nucleic acids with Mvo10b may prevent the binding of protein on the column. Therefore, only the precipitation of nucleic acids with PEI is the most effective method for obtaining purified Mvo10b.

For precipitation of nucleic acids and unwanted proteins, PEI has the desirable properties of being inexpensive, working well at slightly alkaline pH where proteins are usually stable, and rapidly forming finely divided precipitates which may be centrifuged and removed very easily. The fraction of the total protein precipitated by PEI is dependent on the pH and the ionic strength. Almost no proteins will bind to DNA or to PEI in 1 M NaCl, leaving the proteins in the supernatant [18]. The different fractions of PEI were tested in the purification of Mvo10b. The PEI-treated supernatants were assayed for nucleic acid concentration using agarose gel electrophoresis and for the presence of Mvo10b using SDS–PAGE electrophoresis. Results showed that almost all the nucleic acids were precipitated and Mvo10b was efficiently separated from nucleic acid contamination at the final PEI concentration of 0.8% (w/v). After removal of the nucleic acids, the Mvo10b precipitated by the ammonium sulfate was then washed with 70% saturated $(NH_4)SO_4$ solution to remove the excess PEI before the nickel ion metal affinity chromatography since the remained excess PEI will destroy the binding of nickel ions to the chelating sepharose. The further purification of Mvo10b was achieved by a gel filtration using superdex G75 media. The collected fraction was concentrated and stored for further investigations. The determined A_{280}/A_{260} value for purified Mvo10b was 1.7 (Fig. 3), indicating that Mvo10b was purified successfully without nucleic acid contaminants. The final yield is about 36 mg proteins per liter of LB culture (Table 1) and 20 mg proteins per liter of M9 culture. The homogeneity of recombinant Mvo10b was checked by MALDI-TOF mass-spectrometry (Fig. 4) and 15% SDS–PAGE (Fig. 1, lane 10), and the purity of the recombinant Mvo10b reached 98%. The observed molecular weight of recombinant Mvo10b is 11004.7 D as given by MALDI-TOF mass-spectrometry which matches the predicted molecular weight of 11005.21 D.

The other three members of Sac10b family, namely mesophilic Mma10b, thermophilic Mth10b, and hyperthermophilic Mja10b from *Methanococcus maripaludis S2*, *Methanococcus thermolithotrophicus*, and *Methanococcus jannaschii*, respectively, were also successfully purified using the above described protocol. Therefore, the above developed protocol of purification can be generally used to purify other DNA-binding proteins.

Secondary structure of Mvo10b

The secondary structure of Mvo10b was investigated by the far-UV CD spectroscopy since the far-UV region (195–250 nm) is sensitive to the secondary structure of the protein. The far-UV CD spectrum of Mvo10b at pH 6.0 (Fig. 5) shows absorption minima at about 210 and 220 nm. The overall shape of the spectrum indi-

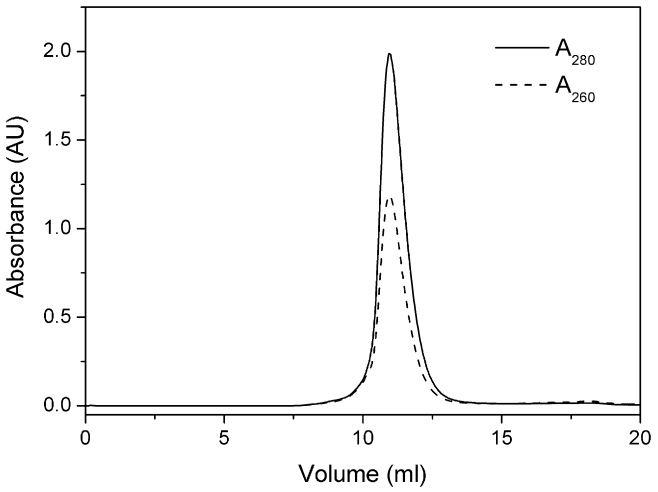

Fig. 3. Superdex G75 (column volume 24 ml) chromatography of Mvo10b purified by the protocol described in this paper. The effluent of each sample was monitored by absorbance A_{280} (solid line) and A_{260} (dashed line) simultaneously.

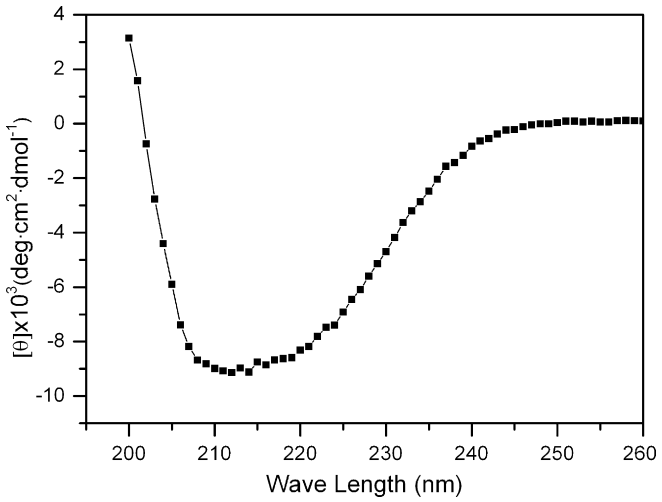

Fig. 5. Far-UV CD spectrum of Mvo10b in 50 mM potassium phosphate buffer (pH 6.0) containing 100 mM KCl.

Table 1
Purification of recombinant Mvo10b[a].

Purification step	Protein (mg)	Recovery (%)
Cell lysate	243	100
After PEI treatment	120	49.4
After $(NH_4)SO_4$ precipitation	80	32.9
After Ni^{2+}-affinity column	54	22.2
After superdex G75 column	36	14.8

[a] These results are obtained from 1 l cell culture.

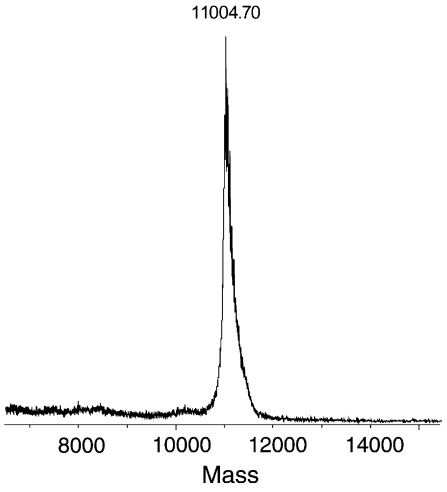

Fig. 4. MALDI-TOF mass spectrum of purified Mvo10b recorded on a Shimadzu AXIMA-TOF^2 mass spectrometry.

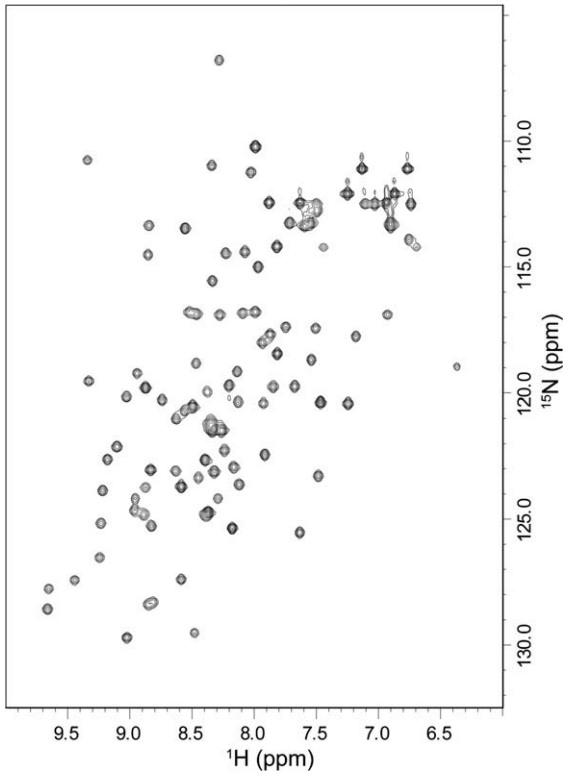

Fig. 6. 2D ^1H–^{15}N HSQC spectrum of ^{15}N-labeled Mvo10b obtained using the procedure developed in this study.

cates the presence also of a certain amount of β-structure. This suggested that Mvo10b has ordered conformation with abundant regular secondary structures. Analysis of the molar ellipticity values provided the estimation of the secondary structural components: 22.9% of α-helix, 25.9% of β-sheet, and 9.9% of turn for Mvo10b in aqueous solution, which are reasonable according to the secondary structural components of [P62A]Ssh10b [11].

Tertiary conformation of Mvo10b

Mvo10b has 90 residues including one proline. The 2D ^1H–^{15}N HSQC spectrum of Mvo10b (Fig. 6) shows a set of well dispersed cross-peaks for almost all the residues in the protein. Meanwhile, all the ^1H_N–^{15}N resonances have narrow and uniform line width. The chemical shifts of ^{15}N and ^1H_N resonances are distributed from about 106.0 to 131.0 ppm and from 6.20 to 9.80 ppm, respectively, in the 2D HSQC spectrum, which is similar to the distribution of cross peaks in the 2D ^1H–^{15}N HSQC spectrum of [P62A]Ssh10b [11]. These reveal that the recombinant Mvo10b has a well folded tertiary structure. The ensemble of NMR and CD data shows that the purified Mvo10b is correctly folded and may have similar 3D structure as its hyperthermophilic counterpart [P62A]Ssh10b.

Moreover, the first ^1H–^1H plane of the 3D ^1H–^{15}N NOESY-HSQC spectrum of Mvo10b shows abundant inter-residue NOE cross-peaks (Fig. 7A), indicating that the recombinant Mvo10b is free

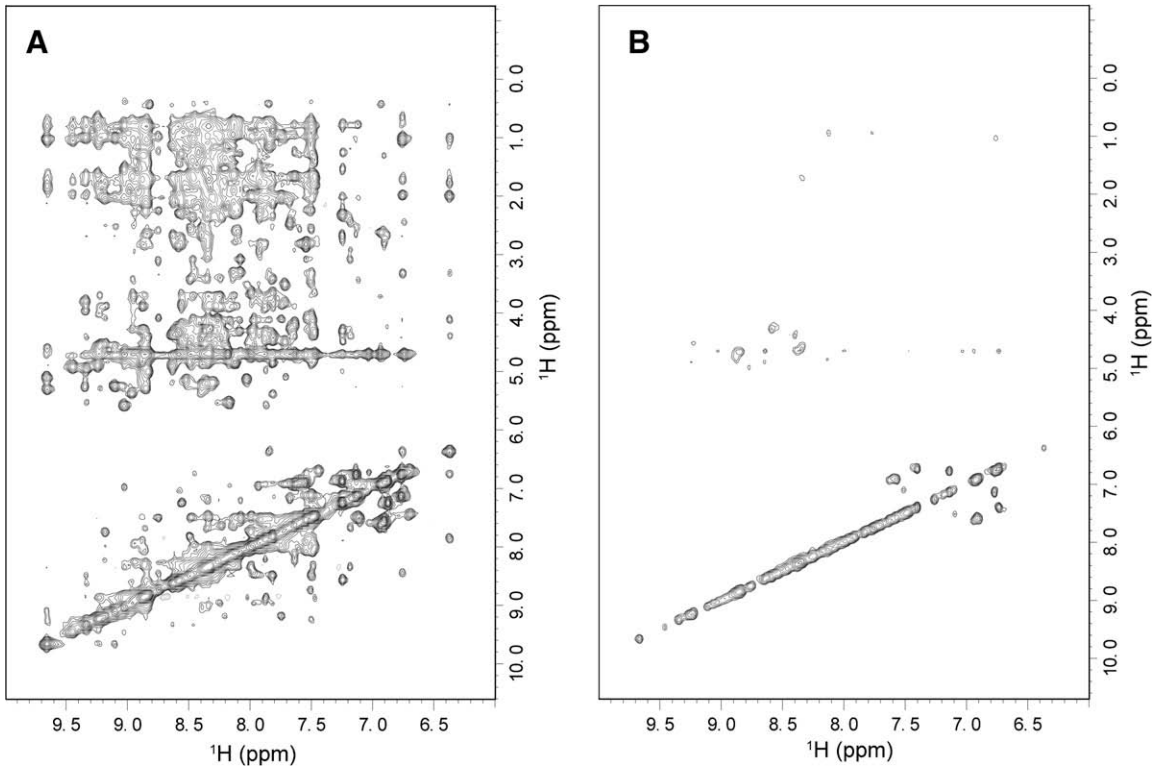

Fig. 7. The first 2D ^{1}H–^{1}H plane of 3D ^{1}H–^{15}N NOESY-HSQC spectrum recorded for Mvo10b. (A) The spectrum of Mvo10b purified by the protocol developed in this study. (B) The spectrum for Mvo10b obtained by a purification protocol similar to protocol for Ssh10b.

of nucleic acid contamination and suitable for the determination of 3D solution structure of Mvo10b by NMR methods. Fig. 7B shows the first ^{1}H–^{1}H plane of the 3D ^{1}H–^{15}N NOESY-HSQC spectrum of Mvo10b purified using the protocol for obtaining recombinant Ssh10b [11]. Only a few NOE cross peaks showed up in this spectrum. Apparently, the Mvo10b isolated following the previous protocol has serious nucleic acid contamination. Therefore, the purification process developed in this study is powerful protocol for isolating the protein from mesophilic archaeon *M. voltae*.

In conclusion, an efficient expression and purification protocol for Mvo10b has been developed. It is possible to utilize this protocol for producing the archaeal Mvo10b in the further research of its structure and function. The protocol is appropriate for isolating the DNA-binding proteins from mesophilic archaeal *M. voltae* and can be also used for the production of the other thermophilic and mesophilic proteins in the Sac10b family.

Acknowledgments

We thank Prof. Li Huang at the Institute of Microbiology, Chinese Academy of Sciences for a generous gift of Mvo10b gene. J.W. was supported by the National Natural Science Foundation of China (NNSFC 30270301) and partially by (NNSFC 30770434).

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