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ARTICLE

One-step purification of histone-like protein (HU) from *Halobacillus litoralis*

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ABSTRACT The histone-like protein (HU) in bacteria is a small, basic, heat-stable protein that is involved in cell division and compression of the bacterial genome into a nucleoid. HU exists as a homodimer in most gram-positive bacteria such as *Bacillus subtilis* and as a heterodimer in enterobacteria such as *Escherichia coli*. The structure of HU, similar to other proteins, may change during purification, which may reduce the value of the investigation. Therefore, in this study, HU was purified in one step using an affinity chromatography column CNBr-activated Sepharose 4B matrix without using high-speed centrifugation and salting out methods. It was observed that the molecular weight and immunochemical properties of HU from *Halobacillus litoralis* were the same as those of HBsu (HU from *B. subtilis*). **Acta Biol Szeged 59(1):19-23 (2015)**

KEY WORDS

Histone like protein
Halobacillus litoralis
affinity chromatography
protein purification

Introduction

HU is a nucleoid-associated, histone-like DNA-binding protein that is found in all gram-negative and gram-positive bacteria. It is highly conserved in prokaryotes (Kamashev et al. 2008), however, it is also encoded in eukaryotic organelles, viruses and bacteriophages (Oberto and Rouviere-Yaniv 1996). HU is a small, basic, heat-stable protein that nonspecifically binds to dsDNA, ssDNA and RNA (Kamashev and Rouviere-Yaniv 2000) and it is involved in regulating DNA supercoiling (Brazdá et al. 2011). In contrast to the nonspecific binding to total cellular RNA and supercoiled DNA, HU specifically recognizes defined structures common to both DNA and RNA. In particular, HU specifically binds to nicked or gapped DNA-RNA hybrids and to composite RNA molecules such as DsrA, a small noncoding RNA (Balandina et al. 2002; Brazdá et al. 2014). In bacteria, HU participates in several major pathways, including control of gene expression, repair, recombination, initiation of oriC-dependent DNA replication (Kamashev and Rouviere-Yaniv 2000), Mu transposition (Kobryn et al. 1999) and compression of bacterial DNA in the nucleoid. HU is important for the optimal survival of cells in the stationary phase and under various stress conditions (Claret and Rouviere-Yaniv 1997; Williams and Foster 2007). *In vitro* studies have shown that similar to histones, HU introduces negative supercoiling into relaxed DNA molecules in the presence of topoisomerase I (Rouviere-Yaniv et al. 1979; Brazdá et al. 2011). *In vivo* studies have shown that HU maintains a DNA superhelical density and modulates topoisomerase I activity (Bensaid et al. 1996). HU regulates the abundance of seqA, a protein involved in preventing re-initiation of chromosome (Lee 2001). Also, mutants defective for HU are more sensitive to UV light and γ-ray irradiation than wild type cells (Boubrik and Rouviere-Yaniv 1995; Li and Waters 1998; Whiteford et al. 2011).

The amino acid sequence of HU is highly conserved among various bacterial species (Drlica and Rouviere-Yaniv 1987). HU deficiency in *Escherichia coli* produces a mild phenotype; however, HU appears to be essential in *Bacillus subtilis* and other gram-positive bacteria (Grove 2011).

HU protein has a specific name in each bacterium, for example, in *B. subtilis* it is called HBsu that is coded by *hbs* gene and is the homolog of HU from *E. coli* (Kohler and Marahiel 1997).Till date, among the *Halobacillus* genus, HU has only been purified from *H. karajensis* using high-speed centrifugation, salting out and ion exchange chromatography (Ghadam and Samadi 2014). This is the first study to use affinity chromatography for HU protein purification in one step.

Materials and Methods

Bacterial strains and culture conditions

Halobacillus litoralis ATCC 700076 [designated as SL-4^T (Type strain) (Spring et al. 1996)] and *Bacillus subtilis* ATCC 6633 were obtained from Iranian Biological Resource Center (Tehran, Iran). *H. litoralis* was cultured in Nutrient Broth (NB; 0.5% peptone, 0.5% sodium chloride, 0.15% beef ex-

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tract and 0.15% yeast extract) and Nutrient Agar (NA; 0.5% peptone, 0.5% sodium chloride, 0.15% beef extract, 0.15% yeast extract and 0.15% agar) with 10% NaCl and 0.5% $MgSO_4$ (pH 7-7.2) at 34 °C. *B. subtilis* was cultured in NB and NA (pH 7) at 30 °C.

Protein extraction

H. litoralis was grown in NB (containing 10% NaCl and 0.5% $MgSO_4$) at 34 °C on a rotary shaker (180 rpm) and harvested by centrifugation (11 000 rpm, 25 min, 4 °C) after 19 h according to *H. litoralis* growth curve. The bacterial pellet was resuspended at 4 volumes of its wet weight in phosphate buffer saline (PBS; 0.8% NaCl, 0.02% KCl, 0.144% $Na_2HPO_4.2H_2O$ and 0.024% KH_2PO_4) pH 7. Then it was incubated with lysozyme (10 mg/ml, Fermentas) at 37 °C for 1 h and sonicated with 9 kHz, 180 W at 0 °C for 5 min (Misonix, NY, USA). Afterwards, suspension was centrifuged at 15 000 rpm for 3 h at 4 °C. The HBsu of *B. subtilis* was also extracted according to the above method.

Enzyme-linked immunosorbent assay

The titration of monospecific polyclonal antiserum anti-HBsu protein (Ghodsi et al. 2010) was obtained by indirect ELISA. It was performed as described by Ghadam and Rabbani (2002). The protein (2-5 µg/ml in PBS) was adsorbed to the surface of the wells (MicroWell MaxiSorp plate, Nunc, Denmark) with overnight incubation at 0 °C. The remaining unadsorbed materials were washed away 4 times by washing buffer (PBS/Tween 20 [0.05% (v/v)]) and the plate were incubated with blocking buffer [Bovine Serum Albumin (BSA) 1% in PBS] for 2 h at 37 °C. The plate was treated with the serial dilution of the specific rabbit anti-HBsu antiserum (3 h at 37 °C). It was further washed and the attached specific antibodies were detected by peroxidase-conjugated goat anti-rabbit IgG (Abcam) diluted 10 000 fold with blocking buffer for 1 h at 37 °C. At the last step, the plate was washed for 4 times with washing buffer and incubated for 20 min at 37 °C with 100 µl substrate solution. This contained equal volumes of TMB (3,3 ,5,5 -tetramethylbenzidine; 0.03 g TMB and 2 ml dimethyl sulfoxide in 100 ml 0.1 M citric acid) and H_2O_2 (0.06% in 0.1 M citric acid). The reaction was terminated by the addition of 50 µl 2 M H_2SO_4 and the color intensity was determined by reading the absorbance at 450 nm.

Protein A-agarose column

The anti-HBsu antiserum was partially purified with 50% saturation concentration of ammonium sulfate and then purified with Protein A-agarose column (Roche). The pH of crude antiserum was adjusted to 7.5-9 with 1 M Tris-HCl (pH 8). The column was washed with 5-10 bed volumes of 100 mM Tris-HCl (pH 8), then it was washed with 5-10 bed volumes of 10 mM Tris-HCl (pH 8) until no protein was detected in the effluent.

The immunoglobulin was eluted with 100 mM glycine (pH 3). To identify the immunoglobulin containing fraction, the absorbance at 280 nm was measured (1 unit OD_{280} = approx. 0.8 mg/ml), then IgG fractions were dialyzed (MWCO 12000, Sigma) against PBS for overnight at 4 °C.

Cyanogen bromide (CNBr)-activated Sepharose 4B beads preparation

The purified anti-HBsu antiserum (1-4 mg/ml) was dialyzed against coupling buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl), then the OD_{280} was measured. CNBr-activated Sepharose 4B beads (Sigma) were swelled in 1 mM HCl for 30-40 min at room temperature. The swelled beads transferred to filter glass funnel and washed with 100 ml of 1 mM HCl. The gel was washed with 10 ml distilled water and 2.5 ml coupling buffer and immediately transferred to antibody solution (gently shaking at 4 °C for 2 h). Afterwards, beads were centrifuged (2500 rpm for 1 min) and the OD_{280} of supernatant was determined. It must be tenfold lower than the OD_{280} of the first antiserum solution. The coupled beads were washed with three folds of total bead volume from coupling buffer and supernatant was separated with 2500 rpm centrifugation for 1 min. Then the beads were incubated with blocking buffer (0.2 M glycine, pH 8) for overnight at 18 °C. The blocked beads were alternatively washed 4 times with coupling buffer (high pH buffer) and low pH buffer (0.1 M acetic acid and 0.5 M NaCl) with checking of the OD_{280} of the first and the last supernatants (OD_{280} should be 0.01 or lower). The gel was poured into column and pre-equilibrated with PBS.

The crude extract was loaded onto a CNBr-Activated Sepharose 4B affinity chromatography column (0.5×1 cm) and was incubated for overnight at 4 °C. The unadsorbed proteins were washed with PBS. Then adsorbed proteins were eluted with 200 mM glycine (in distilled water, pH 2.8). The eluted samples were collected in 20% of total fraction volume from neutralized buffer (3 M Tris-HCl, pH 8.8). All fractions were analyzed with SDS-PAGE.

SDS polyacrylamide gel-electrophoresis

A SDS polyacrylamide gel (15% separating gel, pH 8.8 and 4% stacking gel, pH 6.8) was prepared according to Laemmli (1970). The protein preparations were diluted in 5X sample buffer containing 0.125 M Tris-base (pH 6.8), glycerol, beta-mercaptoethanol and bromophenol blue as tracking dye. The samples were then loaded into the electrophoresis wells after boiling for 5 min. Electrophoresis was carried out in running buffer (0.025 M Tris-base, 0.192 M glycine and 0.1% SDS) at room temperature with constant voltage (120 mV), until

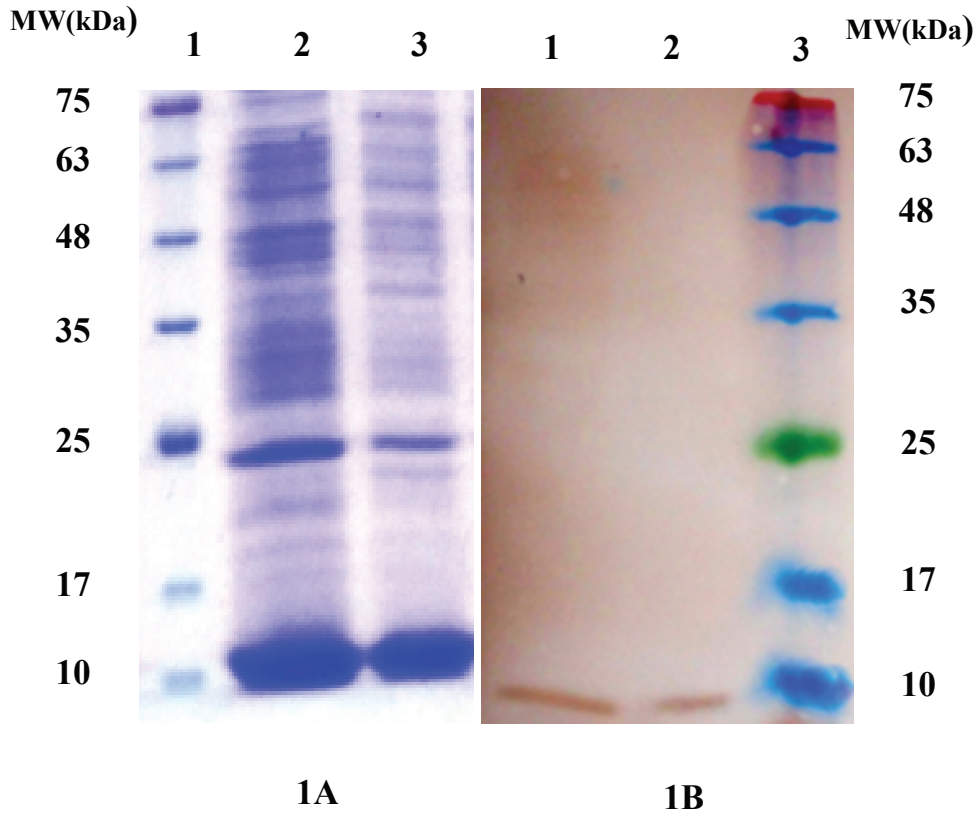

Figure 1A-B. A) SDS-PAGE of the protein extracts stained with Coomasssie Brilliant Blue. Lane 1: protein molecular size marker (Prestained Protein Ladder, 10-180 kDa, SinaClon, Karaj, Iran); Lane 2: protein extract of *H. litoralis*; Lane 3: protein extract of *B. subtilis.* **B)** Immunoblotting with anti-HBsu protein antiserum. Lane 1: protein extract of *H. litoralis*; Lane 2: protein extract of *B. subtilis*; Lane 3: protein molecular size marker (Prestained Protein Ladder, 10-180 kDa, SinaClon, Karaj, Iran).

the tracking dye ran off the gel (Electrophoresis Unit, Akhtarian, Tabriz, Iran). The gel was stained with silver staining (Caetano-Anolles and Bassam 1993) or Coomassie Brilliant Blue R-250 in 50% methanol and 3.5% acetic acid for 30 min, followed by destaining in 10% methanol and 10% acetic acid overnight or until the gel background was clear.

Immunoblotting

Electrophoretically separated proteins were transferred to nitrocellulose membrane (Whatman Protran BA83) in an electrode buffer (25 mM Tris-base, 192 mM glycine, 20% (v/v) methanol, pH 8.3) with 200 mA for 2 h at room temperature. The membrane with the immobilized protein bands was incubated for 1 h at 37 °C with 1% BSA in PBS and washed 3 times for 15 min with PBS. These and all successive incubation and washing steps were performed while solutions were gently shaken. The reaction with the diluted anti-HBsu antiserum (Ghadam et al. 2011) with 1% BSA in PBS (1:1000 diluted) was carried out at 4 °C overnight, followed by the removal of excess antibody by washing with PBS/Tween 20 (0.05%, v/v) for 3 times in 15 min. The membrane was incubated with diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Abcam) with blocking buffer (1:2000 diluted) for 2 h at 37 °C and washed 3 times. Then it was incubated with the substrate solution (1%; 3,3'-Diaminobenzidine, DAB) for 10 min at 37 °C and washed with water for 5 min (Towbin et al. 1979).

Results and Discussion

The DNA-binding protein HU plays an important role in the replication, recombination, and transcriptional regulation (Kamashev and Rouviere-Yaniv 2000). HU is also an efficient mediator of transfection in eukaryotic cells and it is used in drug delivery (Esser et al. 2000) and microchip production (Krylove et al 2001). HU from *Mycobacterium tuberculosis* has been used in crystal structure- and other structure-based drug designing (Koti et al. 2008). Therefore, the purification

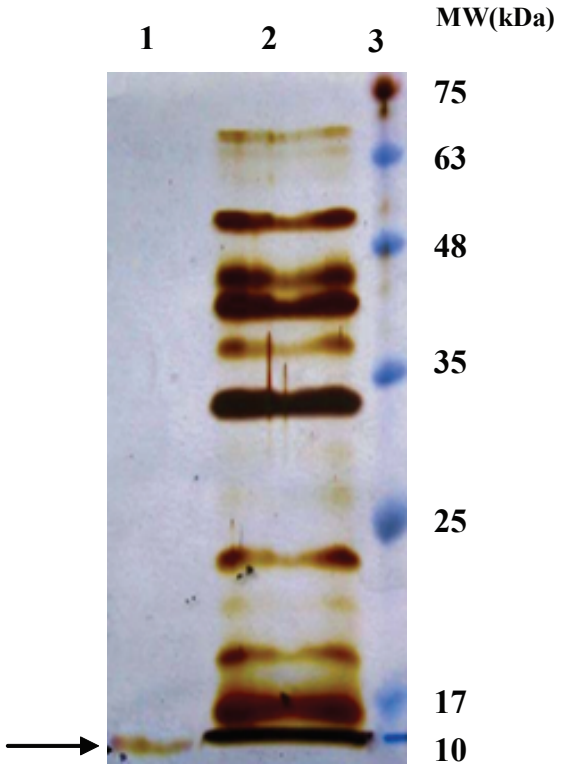

Figure 2. SDS-PAGE analysis of chromatography fractions visualized with silver staining. Lane 1: purified protein; Lane 2: diluted *H. litoralis* crude protein extract; Lane 3: protein molecular size marker (Prestained Protein Ladder, 10-180 kDa, SinaClon, Karaj, Iran).

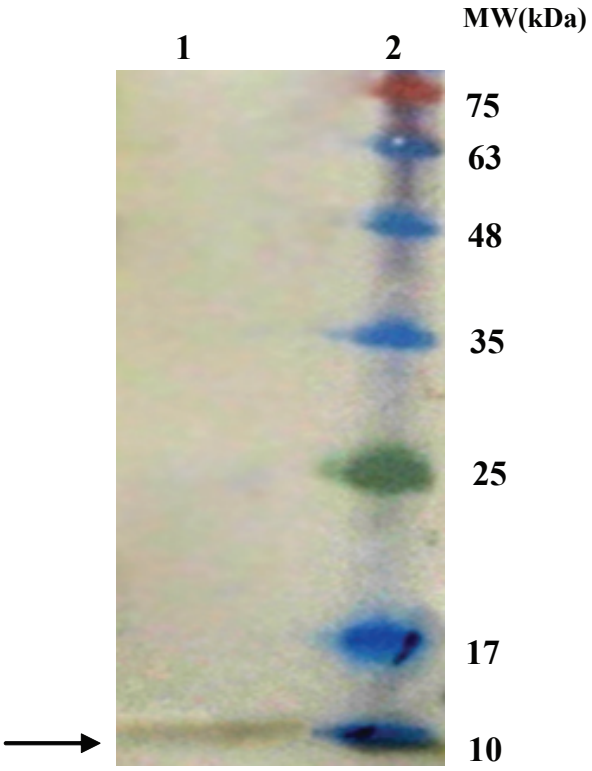

Figure 3. Immunoblotting analysis using anti-HBsu antiserum of the eluted protein from affinity chromatography. Lane 1: fractions eluted with 200 mM glycine buffer; Lane 2: protein molecular size marker (Prestained Protein Ladder, 10-180 kDa, SinaClon, Karaj, Iran).

of this protein from nonpathogenic or archaebacteria can be helpful in drug and gene delivery.

Protein functions are generally destroyed during protein purification steps; so, speed during purification is helpful. In previous studies, we purified HU protein from *Halobacillus karajensis* by using a method combining salting out and ion exchange chromatography (Ghadam and Samadi 2014). In the present study, we purified the HU protein from *H. litoralis* in one-step using affinity chromatography by omitting the salting out step as its accuracy and reproducibility are poor.

There are a few evidences about HU protein in *Halobacillus* bacteria (Ghadam and Samadi 2014), therefore, in this research *H. litoralis* was involved and its HU was compared to that of *H. karajensis*.

In this study, HU from *H. litoralis* and *B. subtilis* was extracted using PBS and lysozyme, and the resultant was analyzed using SDS-PAGE (Fig. 1A) and immunoblotting (Fig. 1B). A single band was observed, indicating that HU from *H. litoralis* is immunologically similar to HBsu from *B. subtilis* and HU from *H. karajensis* (Ghadam and Samadi 2014).

The HBsu antiserum titer was determined using indirect ELISA. The extracted protein was loaded onto analytical affinity chromatography column of CNBr-activated sepharose 4B. The obtained fractions were loaded at 15% SDS-PAGE with modified sample buffer (containing 8 M urea) and stained with silver nitrate (Fig. 2). The single band was immunoblotted to determine the purification accuracy (Fig. 3).

The results showed that the relative molecular weight of the single band from *H. litoralis* on 15% SDS PAGE was approximately 10 KDa, which was similar to that of HBsu and HU from *H. karajensis* (Ghadam and Samadi 2014).

Previous studies have used several steps for purifying HU, such as salting out, high-speed centrifugation and column chromatographies (Ghadam and Samadi 2014). In this study, a new efficient and reproducible method applying only affinity chromatography has been worked out and evaluated for HU purification.

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