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Protein complex purification from *Thermoplasma acidophilum* using a phage display library

Ágnes Hubert [a], Yasuo Mitani [b], Tomohiro Tamura [c], Marius Boicu [a], István Nagy [a,*]

[a] Max-Planck Institute of Biochemistry, Department of Molecular Structural Biology, D-82152 Martinsried, Germany
[b] Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan
[c] Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Sapporo 062-8517, Japan

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ABSTRACT

We developed a novel protein complex isolation method using a single-chain variable fragment (scFv) based phage display library in a two-step purification procedure. We adapted the antibody-based phage display technology which has been developed for single target proteins to a protein mixture containing about 300 proteins, mostly subunits of *Thermoplasma acidophilum* complexes. *T. acidophilum* protein specific phages were selected and corresponding scFvs were expressed in *Escherichia coli*. *E. coli* cell lysate containing the expressed His-tagged scFv specific against one antigen protein and *T. acidophilum* crude cell lysate containing intact target protein complexes were mixed, incubated and subjected to protein purification using affinity and size exclusion chromatography steps. This method was confirmed to isolate intact particles of thermosome and proteasome suitable for electron microscopy analysis and provides a novel protein complex isolation strategy applicable to organisms where no genetic tools are available.

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1. Introduction

Optimal growth conditions of the thermo-acidophilic archaeon *Thermoplasma acidophilum* are around 59 °C and pH 2. Common feature of the genus *Thermoplasma* is that the cells lack a rigid cell wall and are delimited only by a plasma membrane. The genome size of *T. acidophilum* is small (1.5 Mbp) comprising 1507 open reading frames of which 1482 encode for proteins (Ruepp et al., 2000). The lack of cell wall, the relatively small genome, and the low cellular complexity make *T. acidophilum* a favorable model organism for visual proteomics approaches. This approach aims to determine spatial relationships of macromolecular complexes (over 300 kDa) inside an unperturbed cellular environment using cryo-electron tomography and pattern recognition procedures (Nickell et al., 2006).

In the cytosolic proteome of *T. acidophilum* a large proportion of proteins is organized into multimeric complexes, amongst which 35 macromolecular assemblies have been identified with sizes over 300 kDa (Sun et al., 2009). These large protein structures are candidates for single particle analysis using electron microscopy (EM) to create template libraries toward visual proteomics studies and thereby promoting the generation of a comprehensive cellular atlas of macromolecular complexes. Proteomics studies based on 2DE-MALDI-TOF MS approach have provided information on the expressed cytosolic proteins and macromolecular complexes of *T. acidophilum* (Sun et al., 2007). In the protein complement of *T. acidophilum* there are a number of macromolecular assemblies playing important roles in protein folding, degradation, and metabolic pathways, including the archaeal chaperone thermosome (Nitsch et al., 1997), the VCP-like ATPase (VAT), which participates in numerous cellular activities (Gerega et al., 2005; Golbik et al., 1999; Pamnani et al., 1997; Rockel et al., 1999), the 20S proteasome (Zwickl et al., 1999) and the tricorn protease, the core of a modular proteolytic system (Tamura et al., 1996, 1998). Molecular sieve chromatography in combination with LC–MS/MS helped to reveal less abundant cytosolic proteins on the basis of size distribution (Sun et al., 2007). Until now, with the help of single particle electron microscopy and X-ray crystallography, many of these large complexes have been structurally characterized. However, there are still many hypothetical and partially characterized protein complexes whose molecular architecture and biochemical function is not yet explored. The characterization of these macromolecular assemblies could help to elucidate the structure and function of their similar but more complex eukaryotic homologues.

To accelerate the purification of protein complexes novel methods have been developed. Chromosomally tagged protein purification technologies can be used if genetic tools are available for the host under investigation. Tandem affinity purification (TAP) tag is useful for rapid

Abbreviations: EM, electron microscopy; IMAC, immobilized metal affinity chromatography; MS/MS, tandem mass spectrometry; Ni–NTA, nickel–nitriloacetic acid; OHA-300, 300 mM K_2HPO_4–KH_2PO_4 elution fraction of Sup12-HMWF; OHA-500, 500 mM K_2HPO_4–KH_2PO_4 elution fraction of Sup12-HMWF; scFv, single-chain variable fragment; SEC, size exclusion chromatography; Sup12-HMWF, Superose12-separated high molecular weight protein fraction of *T. acidophilum* cytosolic extract; TAP, tandem affinity purification.

* Corresponding author. Tel.: +49 89 8578 2648; fax: +49 89 8578 2641.
E-mail addresses: hubert@biochem.mpg.de (Á. Hubert), mitani-y@aist.go.jp (Y. Mitani), t-tamura@aist.go.jp (T. Tamura), boicu@biochem.mpg.de (M. Boicu), nagy@biochem.mpg.de (I. Nagy).

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purification of complexes from a relatively small number of cells without prior knowledge of the complex composition, activity, or function. This tag can be integrated into the chromosome and TAP-tagged proteins can be expressed and purified using affinity chromatography. One of the key techniques of this method is that the tag sequence contains protease recognition sequence allowing proteolytic release of the bound material under native conditions (Rigaut et al., 1999). This method in combination with mass spectrometry enabled a large-scale approach to characterize multiprotein complexes in *Saccharomyces cerevisiae*, and provided an outline of the proteome as a network of protein complexes (Gavin et al., 2002). However, there are no genetic tools available for many archaea due to limitation in suitable selection markers, marker genes and transformation methods (Baka et al., 2013). Therefore alternative methods like the recently developed grid blotting technology using native polyacrylamide gel electrophoresis combined with EM grid blotting are being used to study protein complexes (Knispel et al., 2012). This is a fast and efficient method to transfer high molecular weight protein complexes from the acrylamide gel matrix directly to EM grids, facilitating structural analysis.

Here we aimed at isolating native protein complexes from cytosolic extract of *T. acidophilum* using a combination of a phage display antibody library and a two-step chromatography method, thereby providing homogenous samples for EM-based structural studies. For this purpose we adapted the mono-antigen targeted phage display technology to a multi-antigen targeted version and generated a combinatorial scFv-library against the high molecular weight fraction of *T. acidophilum*, which contained more than 300 proteins as subunits of protein complexes with sizes larger than 300 kDa. Since protein complexes are sensitive to harsh purification conditions, our goal was to develop an antibody-based mild and fast protein isolation technique. The two-step chromatography method enabled protein complexes to keep their molecular assemblies in their intact and active form for subsequent structural and biochemical analyses.

2. Materials and methods

2.1. Strains and cell culture conditions

T. acidophilum cells were cultured as described earlier with minor modifications (Sun et al., 2007; Robb, 1995). Briefly, 4 mL *T. acidophilum* cryo stock or 1 mL fresh culture was added to 50 mL medium and grown at 59 °C and 120 rpm in an oil bath until OD_{600} reached 1.0–1.2 (2–3 days). Cells were centrifuged at 4.000 ×*g* for 10 min at room temperature (RT), washed with distilled water, and stored at −80 °C. *Escherichia coli* strains TG1 and HB2151 were purchased from Amersham Biosciences and strain BL21(DE3) was from Invitrogen. These strains were routinely cultured at 37 °C in LB (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl), 2× YT (16 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 5 g L^{-1} NaCl) or YTG (16 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 2% (w/v) glucose) media amended with the appropriate antibiotics where needed.

2.2. Preparation of T. acidophilum cytosolic extract

Frozen cell pellet was thawed on ice and resuspended in distilled water (2 mL for 1 g cell pellet) containing EDTA-free protease inhibitor cocktail (Roche). Cell lysis was triggered by elevating the pH of the suspension to 7.5 with non-buffered 1 M Tris. After cell lysis DNase I (Sigma-Aldrich) or Benzonase (Sigma-Aldrich) was added to digest released DNA and RNA and the cell lysate was incubated on ice for 1 h. Crude extract was centrifuged at 30.000 ×*g* and 4 °C for 45 min to remove cell debris and sediment the membrane fraction. Clear cytosolic extract was immediately used or frozen with 15% glycerol and stored at −20 °C.

2.3. Preparation of E. coli cytosolic extract

Ten to fifteen grams of frozen cells were thawed on ice and resuspended in 20 mL milliQ water containing 1/2 tablet of EDTA-free protease inhibitor cocktail (Roche). The cell suspension was supplemented with 1 mg mL^{-1} lysozyme and with the appropriate volume of 10× Coupling buffer (0.08 M Na_2HPO_4, 0.02 M KH_2PO_4, 1.4 M NaCl, 0.1 M KCl, pH 7.4) to obtain 1× buffer concentration and incubated on ice for 1 h. To decrease the viscosity of the lysate 50 μg mL^{-1} of DNase (Sigma-Aldrich) or 2–3 μL of Benzonase (Sigma-Aldrich) was added. The lysate was sonicated on ice five to eight times for 1 min (duty cycle: 30%, output control: 6–8, Sonifier 250, Branson). Crude cell extract was centrifuged at 4 °C, 30.000 ×*g*, 40 min and the supernatant containing the soluble protein fraction was immediately used.

2.4. Column chromatography of macromolecular complexes

Superose12 semi-preparative column (24 mL, GE-Healthcare) was used for the enrichment of macromolecular complexes from *T. acidophilum* cytosolic extract. The column was connected to an FPLC system (ÄKTA Purifier 10, GE Healthcare) and operated at 10 °C. Five-hundred microliters (max. 4 mg protein) of the cytosolic extract was loaded on the column that was equilibrated with running buffer (25 mM potassium phosphate buffer, pH 7.5 containing 1 mM ATP, 1 mM DTT and 5 mM $MgCl_2$) and run at 0.4 mL min^{-1} flow rate. Protein elution profile was monitored with a UV-detector operated at 280 nm. Thirty-five protein-containing fractions of 0.6 mL were collected. High molecular weight protein complex (>300 kDa) containing fractions including the void volume, were pooled and concentrated to 2.5 mL. This sample was mixed with 15% glycerol, aliquoted to 100–200 μL, frozen in liquid nitrogen, named as Sup12-HMWF and stored at −80 °C. Sup 12-HMWF was used as antigen mixture for immunization of mice and for phage biopanning to obtain *T. acidophilum* phage library.

The complexity of Sup12-HMWF was reduced by hydroxyapatite fractionation when it served as protein target mixture to capture specific phages. Sup12-HMWF was loaded on a 20 mL hydroxyapatite column (Bio-Gel HTP, Bio-Rad Laboratories) operated at 10 °C and equilibrated with loading buffer (10 mM potassium phosphate buffer, pH 8.0). Stepwise elution of bound proteins was carried out with 150, 300, and 500 mM potassium phosphate buffers (pH 8.0) at 0.5 mL min^{-1} flow rate. Fractions eluted with 300 and 500 mM potassium phosphate (OHA-300 and OHA-500, respectively) were dialyzed against 20 mM potassium phosphate buffer (pH 8.0) at 4 °C overnight (ON) and concentrated with an ultrafiltration membrane (5 kDa MWCO, Amicon Ultra, Merck Millipore).

2.5. Immunization of mice

Eight Balb/c type 5–6 weeks old female mice were immunized three times with 45 μg of Sup12-HMWF protein mixture in 2 week intervals. The immunization procedure was carried out using Freund's adjuvant as described previously (Harlow and Lane, 1988). Spleens of mice were removed three days after the last injection and stored at −80 °C until use. Immunization and spleen removal of the animals were carried out according to the German Animal Protection Law by the authorized staff of the Animal House of MPI of Biochemistry.

2.6. Construction of scFv repertoire in pCANTAB5E phagemid vector

Seventy mg mouse spleen was subjected to RNA extraction using the RNeasy Midi RNA extraction kit according to the manufacturer's protocol (Qiagen). RNA samples were recovered in 0.5 mL of DEPC-treated water and used immediately as template in RT-PCR reactions to synthesize cDNA. Remaining RNA was stored in 50 μL aliquots at −80 °C. The detailed protocol for library construction in pCANTAB5E phagemid vector (Amersham Pharmacia Biotech) is described in Short protocols in

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