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Purification of ACOD1 expressed in E. coli

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ABSTRACT

This protocol describes the purification of the human enzyme *cis*-aconitate decarboxylase (ACOD1) from recombinant *E. coli* cells.

MATERIALS

All buffers should be vacuum-filtered through a 0.45 µm filter.

Media

ZYM-5052 autoinduction medium, MDAG-135 medium, from Studier et al.

- F. W. Studier, Protein production by auto-induction in high density shaking cultures. *Protein Expr. Purif.***41**, 207-234 (2005).
- F. W. Studier, Stable expression clones and auto-induction for protein production in E. coli. *Methods Mol. Biol.***1091**, 17-32 (2014).

Stock solutions

- 1 M Tris-HCl, pH 8.0, autoclaved

- 5 M NaCl, autoclaved
- 1 M DTT
- 100 mM HEPES pH 7.4 (NaOH)
- 500 mM TCEP, neutralised with NaOH, stored at -20°C

Wash Buffer

- 20 mM Tris, pH 8.0
- 0.5 M NaCl
- 10% (v/v) glycerol
- 1 mM DTT

ElutionBuffer

- 50 mM biotin in Wash Buffer
- pH 8.0 (NaOH)

GF Buffer

- 10 mM HEPES pH 7.4
- 150 mM NaCl
- 10% (v/v) glycerol
- 0.1 mM TCEP

10% PEI, pH 8.0 (HCl)

- MP Biomedicals No. 195444, 50% (w/v) PEI in H_2O. Mw= 50000-100000
- Dilute in water
- Adjust pH to 8.0 with HCl
- Adjust PEI concentration to 10% (w/v)

Reagents and Equipment

- Expression plasmid pCAD29 (pCAD29_hIRG1_4-461_pvp008, Addgene #124843)
- *E. coli* BL21(DE3) CodonPlus-RIL competent cells
- Kanamycin, 100 mg/ml in water
- Chloramphenicol, 34 mg/ml in ethanol
- SOC agar plates
- Lysozyme
- Biotin
- Strep-Tactin XT superflow, 50% suspension (IBA GmbH)
- YMC ECO15/200M0V or similar empty column
- TEV protease
- Syringe filter, 0.45 µm, Sartorius Minisart Plus
- Vivaspin 20, MWCO 30,000 concentrator
- High pressure homogeniser or sonifier. E.g. Avestin EmulsiFlex-C3 homogenizer or Bandelin Sonoplus 2000 Sonifier with a TT13Z probe
- Äkta chromatography machine, 4 ml Strep-Tactin XT column, Superdex 200 26/60 column
- SDS-PAGE equipmentAll buffers should be vacuum-filtered through a 0.45 µm filter.

BEFORE START INSTRUCTIONS

The expression plasmid pCAD29 (pCAD29_hIRG1_4-461_pvp008, Addgene #124843) is used. It contains the coding sequence of human ACOD1, amino acids 4-461, with an N-terminal Streptag and TEV protease cleavage site. The plasmid has a T7 promoter, which is induced by autoinduction here. The method and the plasmid have been described by Chen et al. (F. Chen *et al.*, Crystal structure of cis-aconitate decarboxylase reveals the impact of naturally occurring human mutations on itaconate synthesis. 2019. *PNAS* 116, 20644-20654).
The ACOD1 protein is purified from the cell lysate using Strep-Tactin XT affinity chromatography. A buffer containing 50 mM biotin is used for elution. Attaching the elution buffer with small size tubing to the chromatography system saves costly biotin. The volume for washing the pump with elution buffer can also be minimized, e.g. 5 ml is sufficient on an ÄKTAprime system.
For a good overexpression of the enzyme, it is important that aeration of the autoinduction cultures is sufficient and that the *E. coli* cells grow to high density. Polyethyleneimine (PEI) is added to the cell lysates for precipitation of nucleic acids. This leads to a better separation of insoluble material by centrifugation and seems to improve reproducibility and yield of the process. A yield of 5-10 mg can be expected.

Protein expression

1d

1 Transform BL21(DE3) CodonPlus-RIL *E. coli* cells with the plasmid pCAD29. Plate the cells on a SOC agar plate containing 30 mg/L kanamycin and 34 mg/L chloramphenicol and incubate at 37°C overnight. 1d

2 Inoculate 20 ml MDAG-135 containing 100 mg/L kanamycin and chloramphenicol with a colony and shake over night at 37°C. 1d

3 Prepare 4×1 L ZYM-5052 medium with 100 mg/L kanamycin and 34 mg/L chloramphenicol in 2.8 L baffled Fernbach flasks. Inoculate each Fernbach flask with 4 ml overnight culture and shake 24 h at 130 rpm and 25°C. Ensure sufficient aeration of the flasks, do not close them too tightly. 1d

4 Check the OD_{600} of the culture, 12-14 is expected. Dilute the cells 1:20 in water before measurement. Harvest the cells by centrifugation for 20 min at 5000 rpm. Discard the supernatant. Weigh the cell pellets. Up to 100 g wet cell mass is expected. Using a silicone spatula, transfer the cells to a 500 ml beaker. Rinse the centrifugation flasks and the spatula with Wash Buffer. Add the same volume of buffer as the cell volume. Place the beaker on ice. Resuspend the cells with a magnetic stirrer in a cold room. Transfer the cell suspension to 50 ml 2h

polypropylene tubes, freeze in liquid N2 and store at -80°C.

Preparations for purification

5 Prepare a 4 ml Strep-Tactin XT column. Use an empty YMC ECO15/200M0V or similar column. Prepare buffers and 10% PEI.

Cell lysis

6 Thaw up to 100 g cell suspension in warm water. Transfer to a 500 ml beaker before thawing is complete. Mix the suspension with a magnetic stirrer. Place on ice when thawing is complete. 1h

7 Increase the volume to 250 ml with Wash Buffer. Lyse the cells by sonication or with a high-pressure homogenizer.

8 Sonication with a Bandlin Sonoplus 2000 Sonifier and a TT13FZ probe: 30m

8.1 Place the beaker with the cells on ice. Immerse the probe half way, about 2 cm, into the cell suspension. Set the amplitude to 58%. For 30 min, run cycles of 1 s sonfication and 9 s pause. 30m

9 Remove 2 µl lysate, mix with 8 µl H2O and 4 µl 4×SDS-PAGE sample buffer

Clearing the lysate 1h 30m

10 Add 13 ml 10% PEI per 100 g cells (add less PEI for less cells). Mix, place on ice 15m

11 Centrifuge for 45 min at 4°C and 16,500 rpm or higher speed. Repeat if supernatants are not clear. Collect the supernatants. 45m

12 Remove 2 µl lysate, mix with 8 µl H2O and 4 µl 4×SDS-PAGE sample buffer.

13 Using a 50 ml syringe, filter the supernatant through 0.45 µm syringe filters. 30m

Strep-Tactin chromatography

14 Chromatography can be done at room temperature with an ÄKTAPrime Plus. Mount the 4 ml StrepTactin XT column on an Äkta chromatography system. Equilibrate the column with Wash Buffer 2h

15 Apply the cleared cell lysate to the column at 3 ml/min. Place the lysate bottle on ice. 2h

16 Wash the column with 30 ml Wash Buffer at 3 ml/min. 10m

17 Equilibrate the pump with 5 ml Elution Buffer. Elute the protein with 15 ml Elution Buffer at 1 ml/min, collecting 2 ml fractions. 10m

18 Analyse the lysate aliquots, the flow through and the elution fractions by SDS-PAGE. Determine the protein concentration in the fractions by A280 measurement. 1h

TEV digest

19 Pool the peak fractions, add 1 mg TEV protease and mix gently. Incubate in a refrigerator overnight. 16h

Size exclusion chromatography and concentration 1d 5h 30m

20 Transfer the protein into 2 ml microcentrifuge tubes and centrifuge for 30 min at maximum speed. Pool the supernatants. 30m

21 Compare the protein size before and after TEV by SDS-PAGE, loading 100 ng protein. 1h

22 Equilibrate a Superdex 200 26/60 column with GF Buffer. Apply the protein to the column with a 10 ml sample loop. Elute the protein at 2.5 ml/min and collect 5 ml eluate fractions. 8h

23 Analyse peak fractions by SDS-PAGE. Pool peak fractions and add TCEP to 1 mM. 1h

24 Concentrate the protein with a Vivaspin 20, MWCO 30,000 concentrator (Sartorius), as needed. Aliquot the protein in 200 µl PCR tubes, freeze in liquid nitrogen and store at -80°C. 3h