Py-ADA

PDB:2AMX

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:
Entry Clone Source:
SGC Clone Accession:
Tag:N-Terminal hexahistidine tag with integrated thrombin protease cleavage site: mgsshhhhhhssglvpr*gs(M)
Host:

Construct

Prelude:
Sequence:

mgsshhhhhhssglvprgsEIKFLKKEDVQNIDLNGMSKKERYEIWRRIPKVELHCHLDLTFSAEFFLKWARKYNLQPNMSDDEILD
HYLFTKEGKSLAEFIRKAISVSDLYRDYDFIEDLAKWAVIEKYKEGVVLMEFRYSPTFVSSSYGLDVELIHKAFIKGIKNATELLNN
KIHVALICISDTGHAAASIKHSGDFAIKHKHDFVGFDHGGREIDLKDHKDVYHSVRDHGLHLTVHAGEDATLPNLNTLYTAINILNV
ERIGHGIRVSESDELIELVKKKDILLEVCPISNLLLNNVKSMDTHPIRKLYDAGVKVSVNSDDPGMFLSNINDNYEKLYIHLNFTLE
EFMIMNNWAFEKSFVSDDVKSELKALYF

Vector:p28-LIC-Thrombin

Growth

Medium:
Antibiotics:
Procedure: Py-ADA was expressed in E. coli BL21 (DE3) CodonPlus-RIL in Terrific Broth (TB) in the presence of kanamycin/chloramphenicol (50 µg/mL and 25 µg/mL respectively). A single colony was inoculated into 10 mL of LB with of kanamycin/chloramphenicol (50 µg/mL and 25 µg/mL respectively) in a 125 mL flask and incubated with shaking at 250 rpm overnight at 37 ºC. The culture was transferred into 100 mL of TB with 50 µg/mL kanamycin in a 250 mL shaking flask and incubated at 37 ºC for 3 hours. The culture was transferred into 2 X 1.8 L TB with 50 µg/mL kanamycin and 0.3 mL of antifoam (Sigma) in 2 L bottles and cultured using the LEX system to an OD600 of 2.5. The culture was cooled to 15 ºC, and isopropyl-1-thio-D-galactopyranoside (IPTG) was added to 0.4 mM, and the culture was incubated overnight at 15 ºC.

Purification

Procedure

The cleared cell lysate was loaded onto a column containing 10 g DE-52 resin (Whatman), and then directly onto a 3 mL Ni-NTA (Qiagen) column. When all the lysate was loaded, the two column system was washed with 20 mL binding buffer. The Ni-NTA column was then washed with 200 mL of Wash Buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 30 mM imidazole, and 5 % glycerol). After washing, the protein was eluted from the Ni-NTA column with 15-20 mL of Elution Buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 250 mM imidazole, and 5 % glycerol). EDTA was added immediately to 1 mM; and DTT was added to 1 mM 15 minutes later, then put them in dialysis cassette (Pierce) for overnight dialysis in 10mM HEPES and 500mM NaCl. The following day they were concentrated using a 15 mL Amicon Ultra centrifugal filter device (Millipore), and took absorbance at OD_{280}. Finally aliquots of the purified protein were labeled and stored at -80°C.

Extraction

Procedure
The culture was harvested by centrifugation and the cell pellet was suspended in 160 mL of binding buffer (50 mM HEPES, pH 7.5, 0.5 M NaCl, 5% glycerol, and 15 mM imidazole) with protease inhibitor (1 mM benzamidine-HCl and 1 mM phenylmethyl sulfonyl fluoride, PMSF) and kept in 50 mL Falcon tubes at Â□ 80 ºC. Before purification, the cell suspension was thawed overnight at 4 ºC. Prior to mechanical lysis, each tube of cell suspension was pretreated with 0.5 % CHAPS and 500 units of benzonase (per 40 mL of resuspended cell pellet) for 40 minutes at room temperature. Then the cells were mechanically lysed with a microfluidizer (Microfluidizer Processor, M-110EH) at approximately 18000 psi. The lysate was centrifuged at 24000 rpm for 20 minutes at 10 ºC.
Concentration:
Ligand
MassSpec:
Crystallization: Purified Py-ADA was crystallized using the hanging drop vapour diffusion method in a 24-well plate. A solution containing 20% (w/v) polyethylene glycol (PEG)3350, sodium cacodylate pH 5.5 , 200 mM Magnesium chloride and 20ul BOG were used as the mother liquor.The protein was co-crystalized by 10mM cobalt chloride and 5mM Deoxy Guanosine. Mother liquor (500 µL) was added to the buffer reservoir, and 1.5 µL protein solution was mixed with 1.5 µL mother liquor on cover slides. The crystals appeared in two days.
NMR Spectroscopy:
Data Collection:
Data Processing: