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Purification of metal-dependent lysine deacetylases with consistently high activity

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ABSTRACT

Metal-dependent lysine deacetylases (KDACs) are involved in regulation of numerous biological and disease processes through control of post-translational acetylation. Characterization of KDAC activity and substrate identification is complicated by inconsistent activity of prepared enzyme and a range of multi-step purifications. We describe a simplified protocol based on two-step affinity chromatography. The purification method is appropriate for use regardless of expression host, and we demonstrate purification of several representative members of the KDAC family as well as a selection of mutated variants. The purified proteins are highly active and consistent across preparations.

1. Introduction

Metal-dependent lysine deacetylases (KDACs, also known as histone deacetylases, EC 3.5.1.98) are enzymes that reverse the post-translational modification of lysine acetylation, by catalyzing the hydrolysis of ε-N-acetyllysine residues in proteins via a conserved mechanism [1–3]. Thousands of acetylated protein sequences have been identified in mammalian cells, and thus are subject to deacetylation by KDACs [4–9]. Properly regulated acetylation and deacetylation have been linked to many biological processes, while aberrant KDAC activity has also been linked to numerous diseases [10,11]. Based on the therapeutic potential of regulating KDACs *in vivo*, research efforts are focused on identifying molecules that inhibit or activate these enzymes [11–14], as well as identifying substrates of specific KDACs [15–19]. KDACs are commonly grouped into several classes, with class I, II, and IV KDACs being metal-dependent, and class III (sirtuins) being NAD-dependent. Metal-dependent KDACs require a divalent metal ion in the active site. While KDACs can utilize different metal ions, activity levels are partially dependent upon which metal is present. In addition, KDAC8 is inhibited by excess zinc which binds to a second site on the enzyme [3]. Over 1000 inhibitors for KDACs have been identified, and several are in clinical trials or have already been approved for therapeutic use [11,12]. Despite high interest in understanding KDAC function, relatively few substrates (i.e. acetylated proteins) have been definitively assigned to a particular KDAC. To accomplish this task, purified KDACs are required for *in vitro* activity assays.

Protocols for recombinant expression and purification of KDAC8 from *E. coli* have previously been reported, but are time-consuming and labor-intensive. Expression is generally done in BL21 *E. coli* or a BL21 derivative strain overnight at reduced temperature. Most of the purification protocols rely at least partially on immobilized metal affinity chromatography (IMAC) of TEV protease-cleavable His_6-tagged KDAC8. This initial purification step is usually followed by removal of the tag and a secondary purification step, often involving anion exchange and/or size exclusion chromatography. These secondary purification steps result in a dilute enzyme prep, which must then be concentrated [3,20,21]. One frequently cited protocol then requires an additional step to chelate metals, resulting in a metal-free preparation of apo-KDAC8. Enzyme stored in this manner must be metalated before being used in experiments allowing control of which metal ion resides in the active site to ensure that activity between different preps are comparable [3].

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Following purification, most protocols require storage at −80 °C in small aliquots to avoid freeze/thawing [3,21]. There is even greater variability when considering protocols for purifying the other KDACs, including varying the expression system, tags, and purification methods [18,22].

Critically, KDACs purified using different methods demonstrate differences in activity with the same substrate. A previous comparison of enzyme activity of KDAC8 purified using different metal affinity chromatography protocols resulted in a four-fold difference in activity [23]. In another report, the catalytic efficiency of KDAC8 purified from insect cells was reported to be 3–5 fold higher than the same enzyme purified from *E. coli* [15], although it is unclear whether this is due to a difference in the enzyme resulting from the two different cell types or an artifact of the different purification protocols. Nevertheless, these differences make it impossible to compare KDAC activity against different substrates across reports, as activity differences could be attributed to either the difference in substrate or the enzyme preparation. Here, we present a robust protocol for expression and purification of metal-dependent KDACs. It is applicable across KDACs and expression systems, and is simpler than previously reported protocols. Most importantly, KDAC activity is highly reproducible, even between preparations from different expression systems.

2. Materials and methods

2.1. KDAC expression in E. coli

Expression of KDACs was modified from a previously reported procedure [24]. pJExpress401 vectors (DNA 2.0) containing codon-optimized genes were obtained to express human KDAC8 (pJExpress-KDAC8), KDAC4 (aa648-1057; pJExpress-KDAC4), and KDAC7 (aa521-942; pJExpress-KDAC7) fused to a tobacco-etch virus (TEV) protease cleavage site and His_6 tag. pJExpress-KDAC4 and pJExpress-KDAC8 were subjected to site-directed mutagenesis to introduce the H976Y mutation in KDAC4 (KDAC4HY) and the H143A mutation in KDAC8 (KDAC8HA). For expression in *E. coli*, plasmids were introduced into BL21(DE3) cells for expression. Cells were grown in LB overnight at 37 °C with shaking at 250 rpm, then diluted 1:100 into 2X YT broth and grown under the same conditions. When cells reached an $OD_{600} = 0.8–1.0$, $ZnCl_2$ was added to a final concentration of 50 μM and expression induced with 1 mM IPTG, followed by an additional 3.5 h of growth at 37 °C. After induction, cells were harvested by centrifugation at 3500 rpm for 20 min at 4 °C. Cells pellets were stored at −20 °C until lysis.

2.2. KDAC expression in insect cells

KDAC6 (a gift from Eric Verdin, Addgene plasmid #13823) [25], KDAC7 (aa521-942), KDAC8, and KDAC8HA were cloned into pFastbac1 (Life Technologies) From pJExpress vectors with the TEV protease cleavage site and His_6 tag. Constructs were transformed into DH10Bac *E. coli* cells to produce bacmids containing KDAC8 [26]. Bacmids were purified and transfected into Sf9 cells using Cellfectin II (Life Technologies) as described elsewhere [27]. Baculovirus from these transfections was amplified in Sf9 cells, then used to infect High Five insect cells in suspension in Express Five SFM (Gibco). At 72 h post-infection, cells were pelleted and frozen at −20 °C until lysis.

2.3. KDAC purification

Similar to a previously reported protocol [24], cells were resuspended in either *E. coli* lysis buffer (30 mM MOPS pH 8.0, 150 mM KCl, 5% glycerol, 5 mM imidazole, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mg mL^{-1} egg white lysozyme, 2 U mL^{-1} DNaseI [New England Biolabs], 1X HALT protease inhibitor [Thermo Scientific]) and incubated with rocking for 30 min on ice or insect cell lysis buffer (30 mM MOPS pH 8.0, 150 mM KCl, 5% glycerol, 5 mM imidazole, 2 mM $MgCl_2$, 1X HALT protease inhibitor [Thermo Scientific]). Typically 10 mL of lysis buffer was used per 1 L *E. coli* culture or 250 mL High Five insect cell culture harvested. Cell suspensions were sonicated five times at 50% amplitude for 10 s (Fisher Scientific Sonic Dismembrator Model 120, 1/8″ probe), followed by 30 s on ice. Lysates were clarified by centrifugation at 27,000×*g* for 20 min at 4 °C.

Clarified lysate was added to TALON cobalt resin (Clontech) equilibrated with column buffer (30 mM MOPS pH 8.0, 150 mM KCl, 5% glycerol, 5 mM imidazole) and incubated on ice for 15 min with rocking (resin bed volume of 1 mL per 10 mL lysis buffer). Resin was pelleted by centrifugation at 700×*g* for 5 min and washed twice with 10 bed volumes of column buffer each time. After final centrifugation, resin was transferred to column housing and washed with an additional 10 bed volumes of column buffer. KDACs were eluted (30 mM MOPS pH 8.0, 150 mM KCl, 5% glycerol, 150 mM imidazole) and collected in fractions. KDAC6 was dialyzed into storage buffer (30 mM MOPS pH 8.0, 150 mM KCl, 25% glycerol) overnight at 4 °C with one buffer change. Following dialysis, tris (2-carboxyethyl)phosphine (TCEP) was added to a final concentration of 1 mM. For other KDACs, TEV protease, expressed and purified as described previously [24], was added (1:25) to fractions containing protein, and the mixture was dialyzed in TEV cleavage buffer (30 mM MOPS pH 8.0, 150 mM KCl, 5% glycerol, 1 mM 2-mercaptoethanol, 0.3 mM EDTA pH 7.0) overnight at 4 °C with one buffer change. This was followed by dialysis into buffer containing 30 mM MOPS pH 8.0, 150 mM KCl, and 5% glycerol overnight at 4 °C with one buffer change. Protein was recovered from dialysis and flowed over TALON resin equilibrated with the final dialysis buffer for secondary purification. Purified KDAC (flow-through) was collected. Glycerol and TCEP were added to final concentrations of 25% and 1 mM, respectively.

Where noted, Ni Superflow resin (Clontech) was used for nickel-based purification instead of cobalt. For experiments using zinc-containing resin, TALON resin was stripped with five bed volumes of 0.2M EDTA pH 8.0 and washed with five bed volumes of dH_2O. Then it was regenerated by flowing ten bed volumes of 50 mM $ZnCl_2$ over the resin, followed by seven bed volumes of dH_2O, three bed volumes of 300 mM NaCl, and seven bed volumes of dH_2O.

2.4. SDS-PAGE analysis

Purified KDACs were loaded onto SDS-PAGE gels made from NEXT gel polyacrylamide solution (VWR Amresco) and run at 150 V for 90–120 min. Protein was visualized by staining with Gelcode blue stain reagent (Thermo Fisher).

2.5. Activity assays

{K-ac}-AMC was commercially obtained (Fluor-de-Lys; Enzo Life Sciences). All other peptide substrates were commercial custom peptide syntheses purified to >95% (Genscript). Fluorescamine assays were performed in assay buffer (30 mM potassium phosphate pH 7.6, 100 mM KCl, 5% glycerol) as described previously [24]. Deacetylation reactions using the Fluor-de-Lys substrate were conducted in the same buffer as above and the assay was conducted as previously described [24]. 100 μM substrate was incubated with either KDAC8 (200 nM), KDAC6-His_6 (50 nM), or KDAC6-GST (20 nM) for 1 h at 25 °C. When noted, excess Co^{2+} (ICP-MS standard quality; Ultra Scientific) was pre-incubated with enzyme prior to the addition of substrate. Commercially obtained KDAC8 was