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HPLC-Based Enzyme Assays for Sirtuins

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Abstract

Sirtuins are class of enzymes that utilize nicotinamide adenine dinucleotide, NAD⁺, to remove various acyl groups from protein lysine residues. They have important biological functions and regulate numerous biological pathways. Small molecules that can modulate sirtuin enzymatic activities are potential therapeutic candidates to treat various human diseases. This protocol describes a High Performance Liquid Chromatography (HPLC)-based method to measure the enzymatic kinetics for SIRT2 and SIRT6's demyristoylase activities, and SIRT5's desuccinylase activity. This method uses peptide substrates that resembles physiological substrates and thus can give more reliable kinetic parameters (K_m and k_{cat} values) for these enzymes. The data obtained are useful for understanding the biological function of sirtuins and developing sirtuin modulators.

Keywords

Enzymatic activity assay; High Performance Liquid Chromatography assay; SIRT2; SIRT5; SIRT6; Demyristoylase; Desuccinylase

1. Introduction

Sirtuins are an interesting class of enzymes that regulate aging and transcription, among numerous other biological processes [1,2]. Initially, they were thought to be NAD⁺-dependent protein lysine deacetylases and exert their biological functions by deacetylating various substrate proteins [3,4]. However, several sirtuins do not have efficient deacetylase activities in vitro and were later discovered to remove acyl groups other than acetyl. For example, SIRT5 can efficiently remove negatively charged succinyl and malonyl groups, while SIRT6 can remove long chain fatty acyl groups [5,6]. The physiological functions of these activities have also been demonstrated. Because of the important biological functions of sirtuins, many laboratories around the world are interested in developing small molecules that can modulate the activities of sirtuins. Enzyme activities assays are important for both understanding the physiological function of enzymes and developing small molecule modulators. Here, we describe a HPLC-based method for measuring SIRT2 and SIRT6's demyristoylase activities and SIRT5's desuccinylase activity.

Primary data from the assay can be fitted to Michaelis-Menten equation to derive K_m and k_{cat} values. K_m represents the substrate concentration at which the reaction rate is half-maximal. K_m also reflects the binding affinity between the substrate and the enzyme binding. A lower K_m value indicates a stronger interaction between the enzyme and the substrate. The k_{cat} value represents the turnover number, which characterizes the number of substrates converted to a product in a given time. Combined, k_{cat}/K_m represents the catalytic efficiency of the enzyme [7,8]. The k_{cat}/K_m value is also referred to the specificity constant as substrates with higher k_{cat}/K_m values are more preferred substrates than those with lower k_{cat}/K_m values. Therefore, measuring the k_{cat}/K_m values can help to understand the physiological function of enzymes. Furthermore, developing small molecule inhibitors of sirtuins typically require the measurement of IC_{50} values (50% inhibition concentration). IC_{50} measurement should be carried out with substrate concentrations around K_m values. Substrate concentrations, being too low or too high, will alter IC_{50} values, making it harder to differentiate effective inhibitors among the candidates.

In these assays, we used histone H3-based peptide sequences (amino acids 4-13) with different acyl groups: myristoylated H3K9 for SIRT2 and SIRT6, and succinylated H3K9 for SIRT5 (Fig. 1) [5,6,9,10]. Both peptide substrates are loaded with two tryptophans at the C-terminals, which facilitate the detection and quantification by UV absorption at 280 nm in HPLC. Then, the area of the product peak divided by the sum of both substrate and product peak areas can tell us how much substrate is converted to product, which can in turn yield the initial reaction rate of the enzymatic reaction.

$$\frac{\text{Peak area of product}}{\text{Peak area of product} + \text{peak area of substrate}} = \text{conversion\%}$$

2. Materials

2.1 General Materials for High Performance Liquid Chromatography UV Assay

1. High Performance Liquid Chromatography system with a UV-Vis detector and an autosampler that can take 96-well plates.
2. C18 HPLC Column (100 Å, 75 mm X 4.6 mm, 2.6 µm) (see Note 1).
3. Buffer A: Millipore purified water or HPLC grade water with 0.1 % trifluoroacetic acid (see Note 2).
4. Buffer B: HPLC grade acetonitrile with 0.1 % trifluoroacetic acid.
5. High-speed centrifuge: Centrifuge that can reach to 15,000 x g.

¹.Place a guard column right before the start of the C18 column to avoid precipitated protein from entering the C18 column.

².Recommend using trifluoroacetic acid, for HPLC, 99.0%.

2.2 SIRT2 Demyristoylase Activity Assay

1. Reaction Buffer: 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM NAD⁺ (see Note 3). To 50 μ L of water, add 1.2 μ L of 1 M Tris-HCl (pH 8.0), 2.4 μ L of 25 mM DTT and 2.4 μ L of 25 mM NAD⁺.
2. Quench Buffer: 200 mM HCl and 320 mM acetic acid in methanol.
3. SIRT2 Enzyme: Prepare 6 μ M of purified SIRT2 (see Note 4) [11]. The enzyme is dissolved in 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10% glycerol (see Note 5).
4. Myristoyl H3K9 Peptide: Dissolve the peptide in water at concentrations 7.5 to 960 μ M (see Note 6).
5. Ice

2.3 SIRT5 Desuccinylase Activity Assay

1. Reaction Buffer: 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM NAD⁺ (see Note 3). To 50 μ L of water, add 1.2 μ L of 1 M Tris-HCl (pH 8.0), 2.4 μ L of 25 mM DTT and 2.4 μ L of 25 mM NAD⁺.
2. Quench Buffer: Water with 10% trifluoroacetic acid.
3. SIRT5 Enzyme: Prepare 30 μ M of purified enzyme (see Note 7) [5]. The enzyme is dissolved in 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10% glycerol (see Note 5).

³. Prepare DTT and NAD⁺ fresh every time.

⁴. SIRT2 is purified in truncated length (aa38-256). The Sirt2 gene is inserted into pET28a vector with N-terminal His6-SUMO tag. The vector is transformed into *E. coli* BL21 cells. The cells are cultured in 2L luria broth with 50 μ g Kanamycin (50 mg/ml stock in H₂O, 1:1000) and 20 μ g chloramphenicol (20 mg/mL stock in ethanol, 1:1000). At OD₆₀₀ of 0.6, 20 μ M isopropyl β -D-1-thiogalactopyranoside (IPTG) is added to induce expression (1M stock in H₂O, 40 μ L of IPTG to 2 L of luria broth). The cells are grown at 15 °C, 200 rpm for 16 hours. The following day, the cells are collected by centrifuging at 4 °C, 8000 rpm for 5 mins, and resuspended in 20 mL of 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol and 1mM PMSF. The collected cells are passed through a cell disruptor at least 3 times to lyse the cell. After removing cellular debris by centrifugation at 4 °C, 20,000 rpm for 30 mins, the supernatant is loaded onto a nickel column, pre-equilibrated with 20 mM Tris-HCl (pH 8.0) and 500 mM NaCl. Solution of Imidazole is added in increasing concentration, from 0 to 500 mM, to elute out the desired protein. 1.5 mL of each imidazole solution was used to elute different protein fractions. Each fraction is analyzed by SDS-PAGE to determine whether the desired protein is present. Fractions comprising the desired protein are collected and added into a dialysis tubing. Then 0.1 mL of ULP1, a SUMO protease purchased from commercial source, was added to remove the SUMO tag on SIRT2. The dialysis tubing is incubated at 4 °C overnight with 20 mM Tris-HCl (pH 8.0) and 500 mM NaCl. the protein is additionally purified through a Superdex 75 column (Biorad), using 20 mM Tris (pH 8.0) and 500 mM NaCl. The desired fraction is then concentrated, and 50 % glycerol is added accordingly to make the final glycerol concentration to be 10 %. After the purification, the protein is stored at -80 °C for future use [11].

⁵. Avoid repetitive freeze-thaw. Aliquot the purified enzyme into smaller volumes, so that the left-over enzyme can be discarded after the experiment. The sirtuin enzyme loses activity significantly after several freeze-thaw actions. Store all the enzyme at -80 °C before the experiment.

⁶. The peptides used in this protocol can either be synthesized by a solid-phase peptide synthesizer or purchased from custom peptide synthesis companies. The sequence for myristoylated H3K9 peptide used for this assay is KQTAR-(Myr-K)-STGGWW. The sequence for succinylated H3K9 peptide is KQTAR-(Succ-K)-STGGWW. To validate the concentration, NanoDrop UV-Vis spectrophotometer can be used. The substrate concentration is recommended to be approximately between 0.2 and 5 equivalent of K_m value. In between the interval, more samples with different concentrations are preferred for more accurate measurement. All experiments should be done in triplicate [11].

⁷. SIRT5 is purified in truncated form (aa34-302). It is inserted into pET28a vector with N-terminal His6 tag and expressed in *E. coli* BL21 pRARE2 strain. The cells are cultured in 2L luria broth media at 37 °C until the OD₆₀₀ reaches approximately 0.5. Then, 0.2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) is added to induce protein expression at 15 °C for 16 hours. The cells are harvested by centrifuging at 4 °C, 8000 rpm for 5 mins, and re-suspended in 20 mL of 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol, and 1mM PMSF. Then, the cells are lysed by a cell disruptor three times. The cellular debris is removed by centrifuging at 4 °C, 20,000 rpm for 30 mins. The supernatant from the lysate is loaded onto a nickel column, pre-equilibrated with 20 mM Tris-HCl

4. Succinyl H3K9 Peptide: Dissolve the peptide in water at concentrations 7.5 to 750 μ M (see Note 6).
5. Ice

2.4. SIRT6 Demyristoylase Activity Assay

1. Reaction Buffer: 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 1mM NAD⁺ (see Note 3). To 50 μ L of water, add 1.2 μ L of 1 M Tris-HCl (pH 8.0), 2.4 μ L of 25 mM DTT, and 2.4 μ L of 25 mM NAD⁺.
2. Quench Buffer: HPLC-grade acetonitrile.
3. SIRT6 Enzyme: Prepare 3 μ M of purified SIRT6 enzyme (see Note 8) [6]. The enzyme is dissolved in 20 mM Tris-HCl (pH 7.2), 500 mM NaCl, and 10% glycerol (see Note 4).
4. Myristoyl H3K9 Peptide: Dissolve the peptide in water at concentrations 10 to 1280 μ M (see Note 6).
5. Ice

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 SIRT2 Demyristoylase Activity Assay

This method involves incubating SIRT2 with varying concentrations of myristoyl H3K9, its natural substrate, for a certain period to ensure that the assumption for the Michaelis-Menten equation, $[S] \approx [S_0]$, is met [12]. The reaction is then stopped with the quench buffer and centrifuged vigorously to remove SIRT2 precipitate. The purpose of removing SIRT2 precipitate is to avoid blockade in the HPLC system, which will be used to detect and quantify myristoyl and free H3K9 peptide.

1. Thaw the purified SIRT2 on ice.
2. Prepare the reaction buffer in a 1.5 mL centrifuge tube.
3. Add 2 μ L of purified SIRT2 to the reaction buffer.
4. As soon as the timer starts, add 2 μ L of the myristoyl H3K9 peptide with the given concentration.
5. Vortex briefly (~2 seconds) to mix.

(pH 8.0), 500 mM NaCl and 10% glycerol. Solution of Imidazole is added in increasing concentration, from 0 to 500 mM, to elute out the desired protein. 1.5 mL of each imidazole solution was used to elute different protein fractions. To check the quality of purified protein, SDS-PAGE gel is used. The protein is further purified by Superdex 75 column (Biorad), using 20 mM Tris-HCl (pH 8.0), 500 mM NaCl and 10% glycerol. The desired fraction is then combined and concentrated. The protein is stored at -80°C for future use [5].

⁸SIRT6 is purified in full-length form. It is inserted into pET28a vector with N-terminal His6 tag and expressed in *E. coli* BL21 pRARE2 strain. The cell culture and protein purification use the same method as SIRT5 purification described above in Note 7.

6. Centrifuge the tube for less than 3 seconds, using a table top centrifuge (see Note 9).
7. Place the tube at 37 °C using dry block incubator for 3 minutes (see Note 10).
8. After 3 minutes, remove the tube from the incubator, and add 60 µL of the quench buffer.
9. Vortex the tube thoroughly for 5 seconds (see Note 11).
10. Place the tube on a tube rack.
11. Repeat from #1 for different concentrations of the peptide (see Note 12).
12. Centrifuge all the tubes at 15,000 x *g* for 10 minutes to remove the precipitate SIRT2. The supernatant will be used for HPLC analysis (see Note 13).

3.2 SIRT5 Desuccinylase Activity Assay

This method involves incubating SIRT5 with varying concentrations of succinyl H3K9, its natural substrate, for a certain period to ensure that the assumption for the Michaelis-Menten equation, $[S] \approx [S_0]$, is met [12]. The reaction is then stopped with the quench buffer and centrifuged vigorously to remove SIRT5 precipitate. The purpose of removing SIRT5 precipitate is to avoid blockade in the HPLC system, which will be used to detect and quantify succinyl and free H3K9 peptide.

1. Thaw the purified SIRT5 on ice.
2. Prepare the reaction buffer in a 1.5 mL centrifuge tube.
3. Add 2 µL of purified SIRT5 to the reaction buffer.
4. As soon as the timer starts, add 2 µL of the succinyl H3K9 peptide with the given concentration.
5. Vortex briefly (~2 seconds) to mix.
6. Centrifuge the tube for less than 3 seconds, using a table top centrifuge (see Note 9).
7. Place the tube at 37 °C incubator for 4 minutes (see Note 10).
8. After 4 minutes, remove the tube from the incubator, and add 60 µL of the quench buffer.
9. Vortex the tube thoroughly for 5 seconds (see Note 11).
10. Place the tube on a tube rack.
11. Repeat from #1 for different concentrations of the peptide (see Note 12).

⁹Spin down very briefly. Spending too much time spinning down will cause loss of activity of the enzyme.

¹⁰The enzyme concentration, peptide concentration and reaction time can vary based on the activity of the purified enzyme. The target conversion is less than 10% at all substrate concentrations. This is to make sure that the assumption for the Michaelis-Menten equation, $[S] \approx [S_0]$ is met [12].

¹¹This is to precipitate out all the enzyme in the tube.

¹²Peptide concentrations should vary depending on the activity of the sirtuin. The concentrations should be 0.2 to 5 K_m values [12].

¹³Avoid dropping or any physical collision, which could cause any resuspension of the enzyme.

12. Centrifuge all the tubes at 15,000 x *g* for 10 minutes to remove the precipitated SIRT5. The supernatant will be used for HPLC analysis (see Note 13).

3.3 SIRT6 Demyristoylase Activity Assay

This method involves incubating SIRT6 with varying concentrations of myristoyl H3K9, its natural substrate, for a certain period to ensure that assumption for the Michaelis-Menten equation, $[S] \approx [S_0]$, is met [12]. The reaction is then stopped with the quench buffer and centrifuged vigorously to remove SIRT6 precipitate. The purpose of removing SIRT6 precipitate is to avoid blockade in the HPLC system, which will be used to detect and quantify myristoyl and free H3K9 peptide.

1. Thaw the purified SIRT6 in ice.
2. Prepare the reaction buffer in a 1.5 mL centrifuge tube.
3. Add 2 μ L of purified SIRT6 to the reaction buffer.
4. As soon as the timer starts, add 2 μ L of the myristoyl H3K9 peptide with the given concentration.
5. Vortex briefly (~2 seconds) to mix.
6. Centrifuge the tube for less than 3 seconds, using a table top centrifuge (see Note 9).
7. Place the tube at 37 °C incubator for 10 minutes (see Note 10).
8. After 10 minutes, remove the tube from the incubator, and add 40 μ L of the quench buffer.
9. Vortex the tube thoroughly for 5 seconds (see Note 11)
10. Place the tube on a tube rack.
11. Repeat from #1 for different concentrations of the peptide (see Note 12).
12. Centrifuge all the tubes at 15,000 x *g* for 10 minutes to remove the precipitate SIRT6. The supernatant will be used for HPLC analysis (see Note 13).

3.4 HPLC UV Detection and Data Analysis

After the reaction and removing protein precipitate, the reaction mixture is transferred into the HPLC system with a C18 column to detect myristoyl/succinyl and free lysine peptide using the 280 nm UV absorption. Then, by identifying the peak area of corresponding peptides, the conversion rate can be determined, which can be used to calculate the K_m and k_{cat} values.

1. Add 70 μ L of centrifuged reaction sample to a well of a 96-well plate (see Note 14).

¹⁴. This is to ensure that 60 μ L of the sample is injected in to the system. If less volume is placed in the well, the system will take in air with the sample, causing pressure fluctuation.

2. Program the HPLC software to inject 60 μ L of the sample to the system (see Note 15).
3. For SIRT2 and SIRT6, the chromatography gradient was 0% Buffer B for 2 minutes, 0 – 20% Buffer B for 2 minutes, 20 – 40% Buffer B for 13 minutes, 40 – 100% Buffer B for 2 minutes, and then 100% Buffer B for 5 minutes. The flow rate for SIRT2 and SIRT6 was 0.5 mL/min. For SIRT5, the gradient was 0% to 50% Buffer B for 20 minutes with a flow rate of 1 mL/min (see Note 16).
4. UV-Vis detector was set to measure at wavelength 280 nm.
5. To locate the product or substrate peptide peaks, run a sample only with the standard product or substrate peptide under the same chromatography gradient condition.
6. Integrate the unmodified and myristoyl or succinyl H3K9 peptide peaks (see Note 17).
7. Divide the peak area of unmodified to the total peak area of unmodified and myristoyl or succinyl H3K9 peptide to obtain the conversion rate for each substrate concentration.
8. Multiply the conversion rate with the corresponding substrate concentration, and divide by the reaction time to obtain the initial reaction rate V_i .
9. Plot V_i versus substrate concentration $[S_0]$.
10. Use software such as KaleidaGraph or SigmaPlot to fit the plot to the Michaelis-Menten equation $V_i = V_{\max} * [S_0] / (K_m + [S_0])$ to obtain $V_{\max}$ and K_m . $V_{\max}$ divide by enzyme concentration $[E_0]$ will give k_{cat} .

References

1. Jing H and Lin H (2015) Sirtuins in Epigenetic Regulation, Chemical Reviews. 115, 2350–2375. [PubMed: 25804908]
2. Imai S and Guarente L (2010) Ten years of NAD-dependent SIR2 family deacetylases: implications for metabolic diseases, Trends in Pharmacological Sciences. 31, 212–220. [PubMed: 20226541]
3. Imai S, Armstrong CM, Kaerberlein M, Guarente L (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase, Nature 403: 795–800 [PubMed: 10693811]
4. Sauve AA, Wolberger C, Schramm VL, et al. (2006) The biochemistry of sirtuins, Annual Review of Biochemistry. 75, 435–465.
5. Du J, Zhou Y, Su X, et al. (2011) Sirt5 Is a NAD-Dependent Protein Lysine Demalonylase and Desuccinylase, Science. 334, 806–809. [PubMed: 22076378]
6. Zhang X, Khan S, Jiang H, et al. (2016) Identifying the functional contribution of the defatty-acylase activity of SIRT6, Nature Chemical Biology. 12, 614–620. [PubMed: 27322069]
7. Marangoni AG (2002) Enzyme Kinetics, John Wiley & Sons, Inc., Hoboken, NJ, USA.

¹⁵-It is recommended to only run 1 to 2 samples at a time. The fatty acyl peptide can precipitate out, being absorbed by the plastic after long period of time and making the measurement inaccurate.

¹⁶-For every run, keep an eye on the pressure level. The expected pressure for the experiment is less than 1250 psi. If it is over 1250 psi or much lower than 50 psi, there could be either blockade or leakage in the system. Also, under pump failures, the pressure could fluctuate widely. These would cause inaccurate readings of the UV trace.

¹⁷-Typically, the unmodified H3K9 peptide elutes at retention time of 10.0 minutes: succinyl H3K9 peptide at 10.9 minutes, myristoyl H3K9 peptide at 21.3 minutes.

8. Brooks HB, Geeganage S, Kahl SD, et al. (2004) Basics of Enzymatic Assays for HTS, In: Sittampalam GS, Coussens NP, Brimacombe K, et al. (eds.) Assay Guidance Manual, Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda (MD).
9. Teng Y-B, Jing H, Aramsangtienchai P, et al. (2015) Efficient Demyristoylase Activity of SIRT2 Revealed by Kinetic and Structural Studies, Scientific Reports. 5, 8529. [PubMed: 25704306]
10. Chalkiadaki A and Guarente L (2015) The multifaceted functions of sirtuins in cancer, Nature Reviews Cancer. 15, 608–624. [PubMed: 26383140]
11. Jing H, Hu J, He B, et al. (2016) A SIRT2-Selective Inhibitor Promotes c-Myc Oncoprotein Degradation and Exhibits Broad Anticancer Activity, Cancer Cell. 29, 297–310. [PubMed: 26977881]
12. Berg JM, Tymoczko JL, Stryer L, et al. (2002) Biochemistry, W.H. Freeman, New York.

Figure 1.
Two sirtuins peptide substrates used in the HPLC assay.