Chapter 2

DTNB-Based Quantification of In Vitro Enzymatic N-Terminal Acetyltransferase Activity

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Abstract

We here describe a quick and easy method to quantitatively measure in vitro acetylation activity of not only N-terminal acetyltransferase (NAT) enzymes, but acetyltransferases using acetyl-coenzyme A as an acetyl donor in general.

Key words N-terminal acetyltransferase (NAT), Acetyltransferase, Enzyme activity, Enzyme assay, 5,5′-dithiobis-(2-nitrobenzoic acid), Thiol quantification, Thiol detection, Ac-CoA

1 Introduction

N-terminal acetylation is one of the most common protein modifications in eukaryotes [1]. To date, seven different N-terminal acetyltransferases (NatA–NatG) have been shown to be responsible for this modification. NatA–NatE are ribosome associated and conserved from yeast to humans [2]. NatF is localized to the Golgi membrane and acetylates transmembrane proteins in multicellular eukaryotes [3, 4], whereas NatG is acting inside plant chloroplasts [5]. The NATs have been linked to human diseases. Several NAT subunits have been demonstrated to be mostly up-regulated in different cancer types [6] whereas mutations in the catalytic subunit of NatA may lead to intellectual disabilities and Ogden syndrome [7, 8].

Almost every study of the NATs includes some sort of in vitro assaying of enzyme activity, whether it is to determine substrate specificity, activity of mutants, inhibition studies, or others.

Currently, there are a number of methods available for in vitro quantification of N-terminal acetylation. The high-pressure liquid chromatography (HPLC)-based method described by Evjenth et al. [9, 10] is a sensitive method that is compatible with both recombinant enzyme and immunoprecipitated enzymes. However, each HPLC run is rather time-consuming and when facing large-

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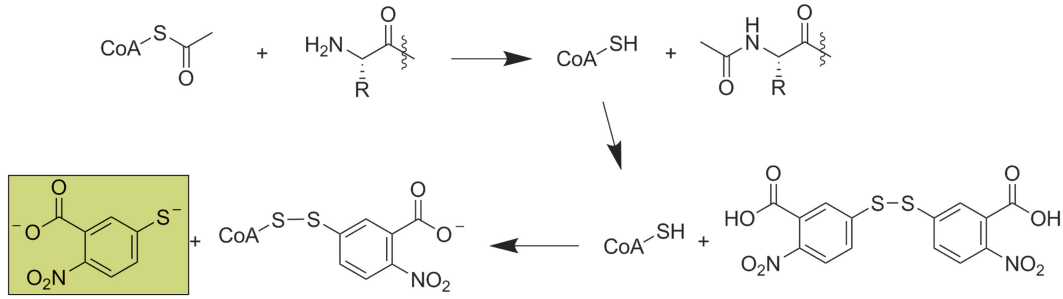

Fig. 1 Ac-CoA donates the acetyl group in the N-terminal acetylation reaction and a thiol is exposed. This thiol is able to readily cleave DTNB that yield TNB^-. TNB^- ionizes to TNB^{2-} in neutral or alkaline pH that yields a light yellow color and absorbs light at 412 nm. As its formation is in 1:1 stoichiometry to acetylated peptide, it can be used as an indirect quantification of N-terminal acetylation

scale or multiple assays it can easily take days and weeks until the samples are analyzed. A radioactivity-based method is another very sensitive method, but requires specialized labs [11].

Thus, there is a need for a fast, safe and cheap method for in vitro quantification of N-terminal acetylation.

As the N-terminal acetylation process involves the transfer of acetyl from acetyl-coenzyme A (Ac-CoA) to the N-termini of peptides, it exposes a thiol-group on CoA. After adding 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), it will readily react with the thiol that yields 2-nitro-5-thiobenzoate (TNB^-) that will ionize to TNB^{2-} in neutral or alkaline pH (Fig. 1) [12]. This is easily quantified by measuring the absorbance at 412 nm.

The only drawback to the method is lower sensitivity compared with other methods and its incompatibility with immunoprecipitated enzyme because of high background probably caused by the cysteine-rich antibodies. However, since the consumption of Acyl-CoA is measured, this method can be used not only for N-terminal acetylation, but for any acylation reaction where Acyl-CoA is consumed.

2 Materials

The acetylation reaction requires purified recombinant enzyme (*see* **Note 1**), a heating block and all samples are analyzed with a spectrophotometer. The samples from our lab are analyzed by an Epoch microplate spectrophotometer from Biotek.

1. 2× Acetylation buffer: 100 mM HEPES-HCl (pH 7.5), 200 mM NaCl, 2 mM EDTA. This is the buffer used in our lab for hNaa50, but most buffers are compatible with the assay as long as they contain as low concentration as possible of reducing agents such as DTT (*see* **Note 1**).

2. Acetyl-CoA: Acetyl-CoA trilithium salt, dissolved in H_2O.
3. Acetyl-acceptor: We use custom-made 24-mer oligopeptides to at least 90% purity, dissolved in H_2O.
4. Quenching buffer: 3.2 M guanidinium-HCl, 100 mM sodium phosphate dibasic (pH 6.8).
5. DTNB buffer: 100 mM sodium phosphate dibasic (pH 6.8), 10 mM EDTA, fresh DTNB is added prior to analysis. 10 mg/mL of DTNB is added yielding a final concentration of around 3 M which is ample for most assays (*see* **Note 2**).

3 Methods

3.1 Substrate Screen

The DTNB assay has many applications, but as an example it may be used to test if an enzyme has enzymatic activity toward a range of specific substrates. In this case, the enzymatic activity of hNaa50 against the N-termini of 24-mer oligopeptides containing the start sequences (one letter amino acid code) MLGP, MLGT, MDEL, and SESS is tested (*see* **Note 3**).

1. Prepare samples with 500 μM substrate peptide and acetyl-CoA, 25 μL 2× acetylation buffer and H_2O up to a total reaction volume of 50 μL. Three replicates are prepared along with two negative controls of each assay condition (toward each substrate peptide). Recombinant hNaa50 is then added to the three replicates to a concentration of 300 nM while enzyme is omitted in the negative controls.
2. Immediately after adding enzyme, quickly vortex the sample and place on a heating block at 37 °C (*see* **Note 4**). The negative controls are also placed on a heating block.
3. After 30 min, stop the assay by adding 100 μL quenching buffer (2-fold volume relative to the reaction volume) and vortex the samples.
4. When all samples are stopped, add the same amount of enzyme to your negative controls as in the positive replicates.
5. Prepare DTNB buffer by adding 5 mg (10 mg/mL) fresh DTNB to 500 μL DTNB buffer (*see* **Note 2**).
6. Add 20 μL of DTNB buffer to each sample and vortex.
7. Of the 170 μL (reaction volume + quenching buffer + DTNB buffer), transfer 150 μL per sample to a microplate before analysis by the spectrophotometer at 412 nm (*see* **Note 5**).

3.2 Quantification of Acetylation

1. Average the negative controls for each substrate peptide and subtract it from the absorbance of the positive replicates.
2. Given that the absorbance has been corrected for path length at the microplate spectrophotometer, l is equal 1 and it is sufficient

Table 1
Absorbance values obtained after acetylation assay with hNaa50 with MLGP, MLGT, MDEL, and SESS oligopeptides

	MLGP	MLGT	MDEL	SESS
Positive replicates	1.268	1.084	1.011	0.961
	1.242	1.121	0.995	0.972
	1.214	1.073	0.987	0.952
Negative controls	0.973	0.983	0.978	0.920
	1.002	0.991	0.997	0.929

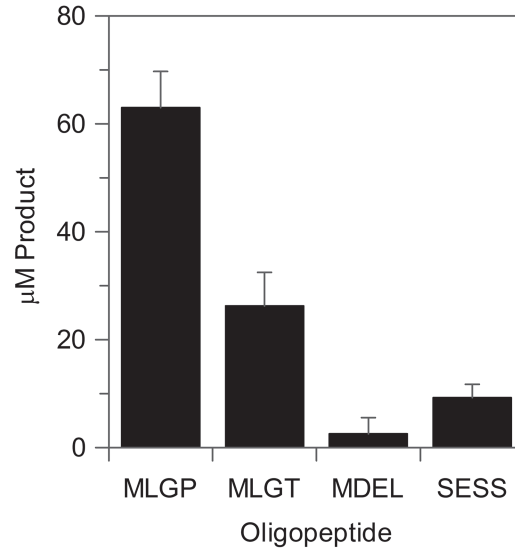

Fig. 2 Using the values in Table 1, the product formation of hNaa50 with MLGP, MLGT, MDEL, and SESS oligopeptides was calculated. The results show high activity toward MLGP (63.0 ± 6.7 μM), some activity toward MLGT (26.2 ± 6.2 μM), and only minor activity toward MDEL and SESS (2.5 ± 3.0 and 9.2 ± 2.5 μM)

to insert ΔAbs and the extinction factor of TNB^- (13,700/M cm) (*see* **Note 6**) into the Lambert Beer equation (Eq. 1)

$$c = \frac{Abs}{\varepsilon * l} \quad \text{(Eq. 1)}$$

3. Multiply the result with the dilution factor (170/50) to obtain the concentration of enzymatic product formation.

Using the values obtained in Table 1, hNaa50 yielded 63.0 ± 6.7, 26.2 ± 6.2, 2.5 ± 3.0, and 9.2 ± 2.5 μM acetylated MLGP, MLGT, MDEL, and SESS oligopeptides respectively (Fig. 2).

4 Notes

1. Recombinant enzymes may be purified in the presence of DTT or other reducing agents as the normally high dilution of enzymes into each sample renders the concentration negligible. Up to 500 μM thiols present may still produce meaningful results although the background noise is significantly increased.
2. Make a stock DTNB buffer (without DTNB) that can be stored at room temperature. When analyzing samples, transfer the amount of buffer needed (20 μL per sample plus a little extra) to a new Eppendorf tube. Subsequently add 10 mg/mL DTNB to the buffer that ensures an ample concentration of 3 M in the final 170 μL sample.
3. The MLGP, MLGT, MDEL, and SESS oligopeptides used as substrates were custom made (Biogenes). The peptides contain seven unique amino acids at their N-termini, as these are the major determinants influencing Nt-acetylation (MLGPEGG, MLGTGPA, MDELDLD, SESSSKS). The next 17 amino acids are essentially identical to the adrenocorticotropichormonepeptidesequence(RWGRPVGRRRRPVRVYP); however, lysines were replaced by arginines to minimize any potential interference by N^{ε}-acetylation.
4. The optimal enzyme concentration, assay temperature, and timeframe must be experimentally determined by timecourse assays. Ensure that the assay setup is within the timeframe of the enzymes linear range. Experience from our lab indicates that it is easier to obtain small standard deviations with product formations of 10 μM and higher, so if experimentally possible, set up the assay conditions to aim for at least 10 μM product formation.
5. Remember to correct for path length at the microplate spectrophotometer.
6. The extinction factor of TNB at 412 nm is 14,150/M cm in phosphate buffer at pH 7.27, but the spectrum is shifted slightly in the presence of guanidinium HCl to 13,700/M cm [13].
7. Being an absorbance-based assay there will be numerical variations in the results. As a test 25 μM CoA was aliquoted into ten samples and analyzed along with ten negative controls (25 μM Ac-CoA). The absorbance measured is given in Table 2. The average concentration measured was 26.26 ± 1.52 μM. Given the small variations, the lower the concentration measured the higher the uncertainty of the data. Thus, to minimize the risk of misinterpreting the data, the assay should be designed in such a way that the sample with maximum activity (e.g., the sample without inhibitor in an IC50 assay) has at least a product formation of 25 μM.

Table 2
Absorbance values obtained after analysis of 25 μM CoA (positive replicates) or 25 μM Ac-CoA (negative replicates)

	Absorbance at 412 nm				
Positive replicates	0.711	0.712	0.715	0.724	0.728
	0.717	0.725	0.713	0.724	0.717
Negative controls	0.623	0.614	0.619	0.613	0.618
	0.628	0.619	0.598	0.603	0.593

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