Journal of Undergraduate Chemistry Research, **2021**, 20(1), 5

DETERMINATION OF ACYL PEPTIDE ENZYME HYDROLASE AND AMINOACYLASE-1 SEQUENTIAL ACTIVITY IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Carson Cole*, Tyler Browning*, Joseph Gilbert*, David Coffman*, Nicolas Drysdale*, Tracy M. Covey†

Department of Chemistry and Biochemistry, College of Science, Weber State University, Ogden, UT 84408

Abstract

Acyl Peptide Enzyme Hydrolase and Aminoacylase-1 are believed to work sequentially to break down N-acetylated peptides. Although there is substantial evidence supporting their consecutive activity, there is also accumulating evidence that both enzymes may have unique roles in various diseases. In this work, we sought to develop an assay that could measure sequential activity of the two enzymes in a biological system. Herein we describe the selection of an acetylated dipeptide that is sequentially hydrolyzed by APEH and ACY1 and detected by gas chromatography-mass spectrometry. This assay offers rapid detection of the amino acid products and was demonstrated to measure the sequential activity of APEH and ACY1 in different biological contexts.

†Corresponding author: tracycovey@weber.edu

Keywords: Gas Chromatography-Mass Spectrometry (GC-MS), Acyl Peptide Enzyme Hydrolase (APEH), Aminoacylase-1 (ACY1), Sequential Enzymes

Introduction:

N-terminal acetylation is one of the most common co-translational modifications and is found on nearly all human proteins. The N-acetylation modification has functional consequences for proteins including stability, function, and translocation effects[1]. Since a high number of proteins are N-acetylated, the enzymes involved in catabolizing these modifications are important for the intracellular recycling of proteins. Two enzymes are involved in the hydrolysis of an N-terminal acetylated peptide: Acyl Peptide Enzyme Hydrolase (APEH) and Aminoacylase-1 (ACY1). APEH acts as an exopeptidase to cleave an N-acetylated (N-Ac) amino acid from the N-terminus and leaves a free polypeptide chain[2,3]. The N-Ac amino acid is then hydrolyzed by ACY1 into a free amino acid and acetate[4]. These two enzymes are found on the same genetic loci and are cytosolic proteins with similar substrate specificity[5]. For example, APEH shows the best kinetic efficiency towards peptides with an N-Ac-Ala, -Met, or -Ser[6]. Likewise, ACY1 hydrolyzes N-Ac-Met, -Ala, or -Ser most efficiently[7]. The activity ratios of APEH and ACY1 in different tissues has been reported to be similar, again suggesting they work together[8]. Furthermore, the substrates for each enzyme act as competitive inhibitors to the other enzyme[9]. To summarize, there is substantial research supporting the biochemical role of APEH-ACY1 in the catabolism of N-Ac peptides.

However, there is a growing body of evidence suggesting that these enzymes have independent roles both physiologically and in disease. APEH appears to have an important role in degrading damaged proteins. APEH catalyzes the hydrolysis and clearance of bacterial N-formylated peptides[10], pyruvylated proteins caused by radical-mediated damage[11], and oxidized proteins[12]. APEH works in conjunction with the proteasome to clear denatured proteins from cells and to resist oxidative stress[13]. APEH also degrades amyloid-beta peptides[14]. APEH enzyme activity is depressed in patients with Alzheimer's Disease[15] and Type II Diabetes[16], which may relate to its role in processing damaged proteins as both diseases are associated with oxidative stress[17,18].

ACY1 is also associated with disease. ACY1 deficiency is an inborn error in metabolism which leads to increased levels of N-acetylated amino acids in the urine[19]. This deficiency is rare and leads to neurological disorders in some patients. ACY1 is reported to be a tumor suppressor in hepatocellular carcinomas[20] and has decreased expression in small cell lung cancers[21]. However, over-expression of ACY1 is found in colorectal carcinomas[22]. It is unclear if either the tumor suppressor or tumor promoting roles of ACY1 relates to its catabolic activity of N-Ac amino acids. The proliferation-promoting effects of ACY1 are suggested to be due to a novel function involving the Sphingosine Kinase type I[23].

Given that the physiological roles of APEH and ACY1 in normal cells and disease states are of interest, we sought to develop an assay that investigates their sequential activity in biological samples. Herein we describe a bioanalytical method to measure the sequential activity through APEH and ACY1. An acetylated Methionine-Alanine (Ac-Met-Ala) dipeptide was selected to be sequentially degraded by APEH-ACY1 and their products are detected by GC-MS. This assay offers rapid detection, quantitative analysis, and can be applied to various biological systems such as cell culture and blood cells. While previous work demonstrates individual enzyme activities, this assay is a useful addition to the field of study to address whether APEH-ACY1 enzymes are working together in various physiological conditions.

Experimental Methods:

Materials:

The following cells were obtained from the ATCC®: A549 (CRM-CCL-185), HeLa (CCL-2™), HCT 116 (CCL-247), HEK-293 (CRL-1573), and BHK (CCL-10). Dipeptide N-Acetylated Methionine-Alanine (Ac-Met-Ala) was synthesized by GenScript (NJ, USA). EZfaast® kits for amino acid analysis by GC-MS were purchased from Phenomenex (CA, USA). Alanine and Methionine standards, and AA74-1 were purchased from Sigma-Aldrich.

Standard Curve Development:

Alanine and Methionine standards were serially diluted in in Tris buffered saline (TBS, pH = 8.0) to concentrations of 1.00 mM, 0.50 mM, 0.25 mM, and 0.125 mM. The standards were prepared in triplicates with the EZ:faast® kit and analyzed by GC/MS. The retention times and peak areas were analyzed with Agilent

MassHunter 2.0. Amino acid values were determined by dividing the amino acid peak area by the internal standard peak area. The internal standard, norvaline, is included in the first step of the EZ:-faast® kit. In these experiments, the internal standard peak area varied some with the final solvent volume, which was affected by length of time on the auto-sampler. Four-point standard curves were completed by graphing the Ala or Met peak area/internal standard peak area vs. concentration (mM). The R2 values for the Alanine and Methionine standard curves were 0.972 and 0.993, respectively.

Measurement of APEH-ACY1 activity:

Cell cultures were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco). Cells were pelleted and lysed with phosphate buffered saline (PBS, pH = 7.4) +1% NP-40 with 1X Halt® protease and phosphatase inhibitors (ThermoFisher Scientific). Whole blood was collected (IRB: 2015-COS-4) in lithium-heparin tubes and separated into erythrocytes. The erythrocytes were lysed hypotonically by adding 100 µL of red blood cells to 600 µL of distilled water and vortexing for 10 seconds. Mouse kidney was obtained from a female C57BL/6 mouse. The frozen kidney was thawed, homogenized, and the cytosolic fraction collected. 50 µg of each cell lysate or 50 µL of erythrocyte lysate was incubated with 5 mM N-Ac-Met-Ala in TBS, pH = 8.0, at 37°C for 0-48 hr. When indicated, 1mM of Zinc Chloride was added to the incubation mixture. Samples were prepared in triplicate with the EZfaast® kit and analyzed by GC-MS.

GC-MS Analysis:

After the derivatization, 50 µL of the organic layer was placed in a GC-MS vial. Gas chromatography (GC) was carried out using the Zebron ZB AA 10 m x 250 µm x 0.25 µm column (Phenomenex) on an Agilent 7890A system. Helium was set to a constant flow at 3.0 mL/min and was heated from 110 °C to 320 °C at a rate of 30 °C*min-1. The Agilent 5975C Mass Spectrometer (MS) was programmed to read molecules anywhere from the 45 g*mol-1 to 450 g*mol-1. MS temperatures were kept at the following temperatures: source at 240 °C, MS quad at 180 °C, and auxiliary at 310 °C.

Results and Discussion

Ac-Met-Ala was selected as an ideal substrate to follow the activity through APEH-ACY1 based on the enzyme specificities[6]. Using this substrate, each enzyme yields a distinct free amino acid product (Figure 1A); APEH hydrolyzes the acetylated N-terminal amino acid from the substrate yielding an acetylated methionine (Ac-Met) and a free alanine (A). ACY1 then hydrolyzes Ac-Met to a free methionine (M). As predicted, Ac-Met-Ala catabolism yields two free amino acids that are separated and detected by GC-MS (Figure 2B). Alanine (A) is first hydrolyzed from the Ac-Met-Ala substrate by APEH and is detected at 1.86 minutes. As Ac-Met accumulates, ACY1 hydrolyzes it to free methionine (M) which is detected at 3.70 minutes. The internal standard (I.S.), norvaline, is detected at 2.45 minutes. Ac-Met-Ala and Ac-Met are not detected by GC-MS, likely because the EZfaast® kit requires derivitization at both the amino and carboxy termini. Attempts to detect substrates and products by GC-MS without derivitization were unsuccessful. Figure 1C illustrates the Ala (A) and Met (M) peaks growing with time. Addition of APEH specific inhibitor, AA74-1, resulted in no detectable peaks (data not shown).

Table 1 summarizes the activity of APEH and ACY1 enzymes, and compares the sequential rate through both enzymes. The activities are given in millimolar of product per minute per microgram of tested sample. These activities are not commensurate with ki-

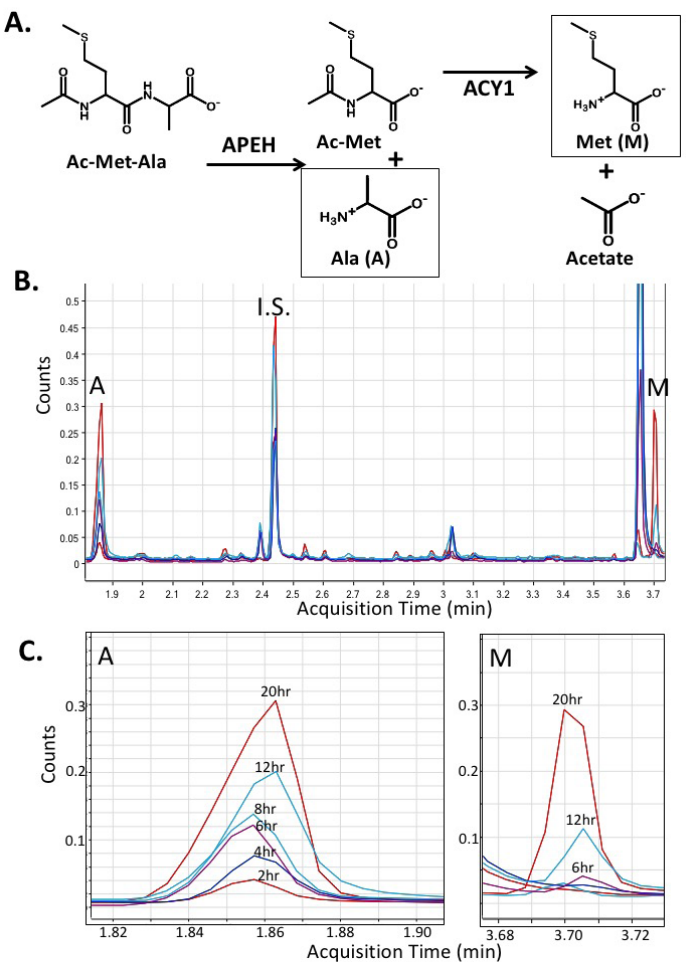

Figure. 1 APEH-ACY1 pathway and product detection.
A. Scheme of APEH and ACY1 hydrolysis of Ac-Met-Ala. Using Ac-Met-Ala as a substrate, each enzyme yields a distinct free amino acid that is detected by GC-MS.
B. GC chromatogram showing separation and detection of Alanine (A), Internal standard norvaline (I.S.), and Methionine (M).
C. Expanded chromatographic window of Alanine (A) and Methionine (M) peaks over time indicating APEH and ACY1 activity respectively

Enzyme Activities in Biological Samples (nM AA/min/µg lysate) *(nM AA/min/µL lysate)	Enzyme Name		
	APEH	ACY1	APEH/ACY1
A549	7.68 ± 0.80	0.09	85.3
HELA	10.1 ± 2.11	N.D.	N.D.
HCT116 (- Zinc)	0.55 ± 0.2	N.D.	N.D.
HCT116 (+Zinc)	1.34	0.39	3.4
Red Blood Cells*	623 ± 44	19.7 ± 4.8	31.7
HEK-293	23.02 ± 6.04	9.97 ± 2.05	2.31
BHK	18.86 ± 1.01	11.33 ± .51	1.66
Mouse Kidney	47.90 ± 4.85	53.32 ± 1.87	0.9

Table 1. Activity of APEH, ACY1, and APEH/ACY1 in various biological samples. The rate is given in nM AA/min/µg lysate (Red blood cells are reported in nM AA/min/µL lysate). APEH/ACY1 ratios near to 1 means that both APEH and ACY1 are active at similar levels in the tested sample. Ratios far from 1 mean that there is variance in APEH and ACY1 measured activity.

netic activities, since it is noted that ACY1 has no substrate available until APEH hydrolyzes the Ac-Met-Ala substrate. However, the goal of this assay is not to measure Michaelis-Menten kinetics but rather to demonstrate APEH and ACY1 sequential activity on a single substrate. The ratio of APEH/ACY-1 denotes how quickly a substrate moves through both enzymes. The lower the ratio, the quicker the substrate moves through both enzymes.

The sequential interaction (APEH/ACY1) varied greatly based on biological context. A549 cells had a high rate of APEH/ACY1 sequential activity, although this is largely due to very low ACY1 activity. A549 are non-small cell lung cancer cells that have been reported to have ACY1 detectable activity[21], however it appears relatively low compared to APEH activity. HeLa and HCT116 cancer cells demonstrated no detectable ACY1 activity over the time course even though detectable APEH activity was observed in both lines. This was surprising since HCT116 cells have been reported to have overexpression of ACY1[22], although notably protein expression and not enzyme activity was tested. Previous publications have shown that APEH activity and expression do not correlate[15,16]; although this has not been investigated for ACY1, it would not be unreasonable to find ACY1 expression and activity are different. Since ACY1 requires a zinc cofactor for activity[24], HCT116 cells were retested in the presence of 1mM ZiCl2 to see if Zinc is a limiting cofactor. Interestingly, addition of zinc cofactor to the cell lysates led to slightly increased activity of both APEH and ACY1 in HCT116 cells. Zinc was not added to other assays since the goal was to test APEH/ACY1 activity under physiological conditions.

The results show substantial variability of APEH, ACY1, and APEH/ACY1 activities across the tested cancer cells, but we also wanted to test normal cells. In human red blood cells, APEH activity is much higher than ACY1 activity. This result is consistent with APEH and ACY1 expression levels in human blood found in BioGPS[25] and reported enzyme activities[5, 21]. APEH and ACY1 are both highly expressed in kidneys[25] and thus it was expected that both enzymes would have high activity. Using three different kidney samples, HEK-293 cells, BHK cells, and a kidney from a B6 mouse, we found easily detectable activity for both enzymes in all kidney samples tested. Notably, the ratio of APEH/ACY1 is fairly low (i.e. close to 1) in the kidney samples, indicating similar activity of these enzymes. The data in table 1 suggests APEH and ACY1 function similar to their published expression levels in normal cells but their activity is much more varied in cancer cells.

Herein we describe a bioanalytical assay to detect sequential enzyme activity through the APEH/ACY1 proteolytic pathway. GC-MS is an effective method to elucidate the non-steady state activity of APEH-ACY1 enzyme pathway in diverse biological samples. The assay described here allows monitoring of enzyme activities, which is a complimentary addition to expression studies of these enzymes. Furthermore, studying the sequential activity of APEH-ACY1 may shed light on differing roles of the enzymes between normal and disease states.

Acknowledgement:

This work was supported by an internal Weber State University undergraduate research grant funded by the Ralph Nye Charitable Foundation.

References

(1) Aksnes, H.; Hole, K.; Arnesen, T. *Int. Rev. Cell Mol. Biol.* **2015**, *316*, 267-305.
(2) Sharma, K. K.; Ortwerth, B. J. *Eur. J. Biochem.* **1993**, *216* (2), 631–637.
(3) Fujino, T.; Tada, T.; Beppu, M.; Kikugawa, K. *J. Biochem.* **1998**, *124* (6), 1077–1085.
(4) Birnbaum, S. M.; Levintow, L.; Kingsley, R. B.; Greenstein, J. P. *J. Biol. Chem.* **1952**, *194*, 455-470.
(5) Perrier, J.; Durand, A.; Giardina, T.; Puigserver, A. *Biochimie* **2005**, *87* (8), 673–685.
(6) Krishna, R. G.; Wold, F. *Protein Sci.* **1992**, *1* (5), 582–589.
(7) Gade, W.; Brown, J. L. *BBA - Enzymol.* **1981**.
(8) Perrier, J.; Giardina, T.; Durand, A.; Puigserver, A. *Biol. Cell* **2002**, *94* (1), 45–54.
(9) Scaloni, A.; Jones, W.; Pospischil, M.; Sassa, S.; Schneewind, O.; Popowicz, A. M.; Bossa, F.; Graziano, S. L.; Manning, J. M. *J. Lab. Clin. Med.* **1992**, *120* (4), 546–552.
(10) Nguyen, K. T.; Pei, D. *Biochemistry* **2005**, *44* (23), 8514–8522.
(11) Yoshioka, K.; Komatsu, T.; Hanaoka, K.; Ueno, T.; Terai, T.; Nagano, T.; Urano, Y. *Chem. Commun.* **2016**, *52* (23), 4377–4380.
(12) Fujino, T.; Watanabe, K.; Beppu, M.; Kikugawa, K.; Yasuda, H. *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* **2000**, *1478* (1), 102–112.
(13) Shimizu, K.; Kiuchi, Y.; Ando, K.; Hayakawa, M.; Kikugawa, K. *Biochem. Biophys. Res. Commun.* **2004**, *324*, 140–146.
(14) Yamin, R.; Zhao, C.; O'Connor, P. B.; McKee, A. C.; Abraham, C. R. *Mol. Neurodegener.* **2009**, *4*, 33.
(15) Palmieri, G.; Cocca, E.; Gogliettino, M.; Valentino, R.; Ruvo, M.; Cristofano, G.; Angiolillo, A.; Balestrieri, M.; Rossi, M.; Di Costanzo, A. *J. Alzheimer's Dis.* **2017**, *60* (3), 1097–1106.
(16) Covey, T.; Marshal, I.; Prince, D.; Johnson, H.; Ruiz, D.; Nicholaou, M. *BIOS* **2018**, *90*(2), 70-78.
(17) Christen, Y. *Am. J. Clin. Nutr.* **2000**, *71*(2), 621S-629S.
(18) Rains, J. L.; Jain, S. K. *Free Radical Biology and Medicine*. *2011*, *50*(5), 567-575.
(19) Sommer, A.; Christensen, E.; Schwenger, S.; Seul, R.; Haas, D.; Olbrich, H.; Omran, H.; Sass, J. O. *Biochim. Biophys. Acta - Mol. Basis Dis.* **2011**, *1812* (6), 685–690.
(20) Wei, X.; Li, J.; Xie, H.; Ling, Q.; Wang, J.; Lu, D.; Zhou, L.; Xu, X.; Zheng, S. *Cancer Lett.* **2014**, *351* (1), 117–125.
(21) Miller, Y. E.; Minna, J. D.; Gazdar, A. F. *J. Clin. Invest.* **1989**, *83* (6), 2120–2124.
(22) Shi, H.; Hayes, M. T.; Kirana, C.; Miller, R. J.; Keating, J. P.; Stubbs, R. S. *Hum. Pathol.* **2013**, *44* (6), 1089–1097.
(23) Maceyka, M.; Nava, V. E.; Milstien, S.; Spiegel, S. *FEBS Lett.* **2004**, *568* (1–3), 30–34.
(24) Lindner, H. A.; Lunin, V. V.; Alary, A.; Hecker, R.; Cygler, M.; Ménard, R. *J. Biol. Chem.* **2003**, *278* (45) 44496-44504.
(25) Su, A. I.; Wiltshire, T.; Batalov, S.; Lapp, H.; Ching, K. A.; Block, D.; Zhang, J.; Soden, R.; Hayakawa, M.; Kreiman, G.; Cooke, M. P.; Walker, J. R.; Hogenesch, J. B. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101* (16), 6062-2067.