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Short communication

High throughput detection of deamidation using S-(5′-adenosyl)-L-homocysteine hydrolase and a fluorogenic reagent

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ABSTRACT

Deamidation of asparagine (Asn) residues is one of the most common chemical degradation pathways observed in proteins. This reaction must be understood and controlled in therapeutic drug candidates, as chemical changes can affect their efficacy and safety. The analytical tools available for detection of deamidation reaction products, such as isoaspartic acid residues, are either chromatographic or electrophoretic, and require MS detection for absolute identification of peaks. High-throughput measurement of protein degradation has typically been limited to probing the target's physical state using spectroscopic techniques. Here, we describe a high throughput assay for isoaspartate residues using fluorescent detection in a microtiter plate format. The method allows for fast detection of protein deamidation in a cost-efficient manner. The method can be employed even if the target peptide or protein contains free Cys residues. The technique appears to be selective, linear, and accurate.

1. Introduction

Therapeutic proteins are the largest class of new drug molecules in development to treat disease [1]. Both efficacy and safety of drug candidates must be demonstrated before a molecule can be marketed. As such, the long-term physical and chemical stability of a protein drug must be ensured. Methodologies for characterization and monitoring of protein drug candidates likewise continue to evolve. High-throughput methods capable of analyzing many samples in a short amount of time are finding increased utility in drug development labs, where efficient candidate screening is necessary to meet demanding timelines. These methods are typically performed on a microtiter plate and forgo traditional chromatographic or electrophoretic separation, instead probing the sample using spectroscopy alone. Fluorescence, UV absorbance, or light scattering can be used alone or combined with thermal ramping to investigate physical properties such as T_m, K_d, size, or viscosity [2–5]. Bommana recently developed a high-throughput assay for detection of oxidation [6]. However, detecting deamidation still requires separation techniques.

Deamidation involves the degradation of an asparagine residue (Asn) to aspartic acid (Asp) or isoaspartic acid (isoAsp). This degradation pathway is commonly observed using methods that discriminate by charge, such as capillary isoelectric focusing (cIEF) or cation exchange chromatography (CEX). While deamidation can be observed this way, the methods are nonspecific to the deamidation reaction. Peptide map HPLC/MS allows for a more specific quantification of deamidation, and pinpoints the location of the change on the peptide chain. While informative, peptide maps are time consuming and not appropriate for rapid sample analysis.

Specific analysis of the isoAsp residue has been achieved by a progression of methods that use the enzyme protein isoaspartyl-methyltransferase (PIMT). This enzyme is found in eukaryotes, where it is used to repair isomerized aspartic acid residues. PIMT transfers a methyl group to the isoAsp acid, converting it to an ester that can then be attacked by the isoAsp non-native backbone nitrogen. This selectivity was first used to detect isoAsp by the transfer of a radiolabeled methyl group to the target residue [7]. This was a labor-intensive method, and required safety precautions for the use of radiolabeled material. A second generation isoAsp assay used PIMT to transfer a methyl group from S-adenosyl methionine (SAM) to the target, creating the byproduct S-adenosyl homocysteine (SAH). SAM and SAH could then be separated and quantified using HPLC [8]. Understanding that isoAsp is a byproduct of deamidation, Carlson and Riggin used similar methodology to examine

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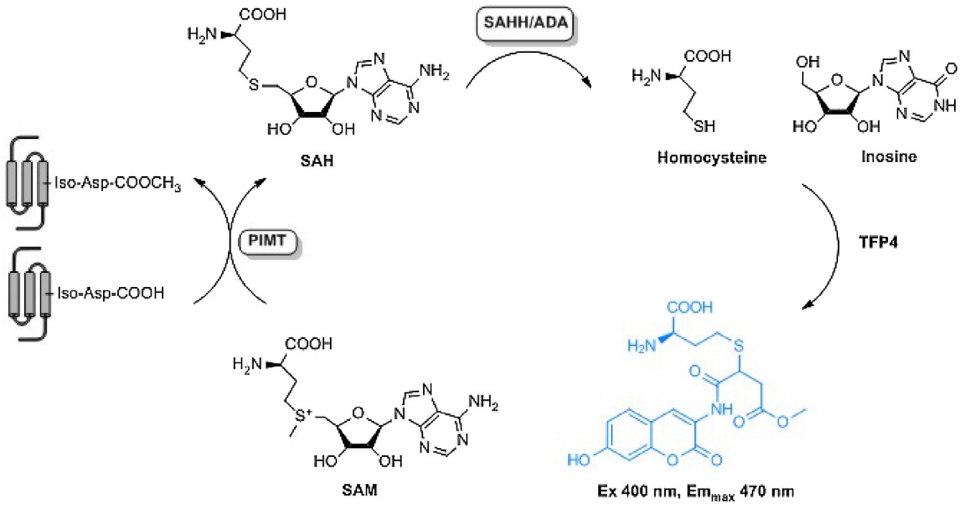

Fig. 1. Reaction schematic for detection of isoaspartate using PIMT, SAHH, and fluorogenic reagent thiol fluorescent probe IV.

deamidation specifically [9]. A separation-based method is currently marketed by Promega (IsoQuant Deamidation Kit), which quantifies SAH using HPLC.

Presented here is a third-generation PIMT assay for deamidation using a coupled enzymatic reaction to hydrolyze SAH to homocysteine, which is then labeled with a fluorogenic reagent (Fig. 1). Fluorescent or colorimetric observation of methyltransferase reactions where SAM is the methyl donor are of interest in the fields of biology and biochemistry [10,11]. Almost universally, researchers have probed this reaction by employing SAH hydrolase (SAHH), which converts the SAH product into adenosine and homocysteine. Burgos et al. and Drake et al. followed this reaction by detecting adenosine in luciferase-based assays [12,13]. A similar approach to quantifying the adenine by-product has appeared, but using different fluorescent labeling chemistry [14]. Hudec et al. and Wang chose to target the homocysteine product, labeling the free thiol with ThioGlo1 and CPM, respectively [15,16]. Similarly, there have been reports of modifying the IsoQuant kits to use fluorimetric detection. This has been called IsoQuant-Glo, but the specifics of the fluorescent label are not provided [17,18]. Here, this enzymatic framework is applied specifically to the detection of isoAsp in a high-throughput assay for deamidation of proteins and peptides using a novel fluorescent compound

2. Materials and methods

2.1. Materials

Protein isoaspartyl methyltransferase (PIMT), isoasp-DSIP reference standard (DSIP), and S-adenosyl-L-methionine were purchased from Promega as part of the IsoQuant Isoaspartate Detection Kit (P/N MA1010). Zeba Spin 7k MWCO desalting columns were purchased from Thermo (P/N 89882). ThioGlo3 (TG3) was purchased from Covalent Associates. Thiol Fluorescent Probe IV (TFP4) was purchased from Millipore (P/N 595504). PolySULFOETHYL A 200 × 4.6 mm, 5 μm, 300 Å cation exchange column was purchased from PolyLC Inc (P/N 204SE0503). Human adenosine deaminase (hADA, P/N 93985), 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM, P/N C1484), DL-homocysteine (Hcys, P/N H4628), and S-(5′-adenosyl)-L-homocysteine hydrolase from rabbit erythrocytes (SAHH, P/N A1705) were purchased from Sigma. Black U-bottom 96 well microtiter plates were purchased from Fisher (P/N 267342).P-Hcy-1 dye was synthesized and purified by Tarik Ozumurzifon. Glucagon (P/N 4074733) was obtained from Bachem. All other chemicals and materials were obtained from Fisher and used as received.

2.2. Reagent preparation

Fluorogenic reagents were solubilized in DMSO prior to dilution in microtiter plate wells. PIMT enzyme was buffer exchanged twice into 20 mM Tris, 1 mM EDTA, 20% glycerol pH 7.5 using Zeba desalting columns under conditions recommended by Thermo. SAHH enzyme was diluted 3-fold in the same Tris/EDTA/glycerol buffer, then buffer exchanged twice into 20 mM Tris, 1 mM EDTA, 20% glycerol pH 7.5 using Zeba desalting columns. hADA was diluted by a factor of 100 into Tris/EDTA/glycerol buffer. SAM was diluted by a factor of 10 in water prior to use. All assay components derived from the ISOQuant deamidation kit and hADA were stored at −20 °C while not in use. All other enzymes and fluorescent reagents were stored at −80 °C while not in use.

2.3. Fluorogenic assay for asparagine deamidation

All assays were performed in black 96-well microtiter plates. A reaction mixture with enough volume for a given number of wells was prepared daily. For each well, the reaction mixture contained 2.5 μL of 1/10 SAM dilution in water (0.1 mM), 2.5 μL purified PIMT enzyme, 0.9 μL diluted and purified SAHH enzyme (~0.01 U/μL), 0.25 μL diluted hADA (0.01 U/mL), 7.5 μL 0.5 M sodium phosphate 5 mM EDTA, and 20.1 μL water for a total volume of 33.75 μL reaction mixture per well. 2.5 μL of sample was added to each well, followed by the addition of 33.75 μL reaction mixture which was mixed with the sample in the well by pipette. The plate was then sealed and incubated for 3 h at 37 °C. Following reequilibration to room temperature, 2.5 μL of 200 μM TFP4 was added to each well and mixed by pipette. The plate was then covered from light and incubated for 10 min to allow the fluorogenic reaction to go to completion. Sample fluorescence was measured using a BioTek Synergy2 plate reader. Excitation was performed at 360 nm with a 40 nm bandwidth, and emission was recorded at 460 nm with a 40 nm bandwidth. Detection was performed with the Top 50% function, and sensitivity was set to 90.

2.4. Glucagon stability study

Glucagon was solubilized in two buffer systems, 20 mM glycine, pH 10 and 20 mM glycine, 100 mM NaCl, pH 10 at a concentration