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A mechanistic analysis of the quantitation of α-hydroxy ketones by the bicinchoninic acid assay

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ABSTRACT

A new class of compounds amenable to quantification by the bicinchoninic acid (BCA) assay was identified, allowing an expansion of compounds quantifiable within the assay's capacity. In this article, we demonstrate that compounds containing the α-hydroxy ketone structure are easily measured under standard BCA assay conditions. A nonchromophore analyte containing the α-hydroxy ketone structure, 1,3-dihydroxypropan-2-one (commonly known as dihydroxyacetone), and various structural derivatives were explored on an equimolar basis in the BCA assay. Combined with earlier studies exploring α-hydroxy ketones within copper oxidation systems, the data support the mechanism of this class of compound's ability to enolize through an enediol intermediate to generate a strong signal in the BCA assay. This new quantification technique also highlights the potential for α-hydroxy ketones to interfere with other analytes quantified by the BCA assay.

The bicinchoninic acid (BCA)[1] assay, originally developed as a way to quantify the concentration of reducing sugars, is widely used as a powerful tool for protein quantitation [1–5]. The BCA assay determines the total concentration of an analyte through the reduction of Cu^{2+} ions under alkaline conditions. This reduction event generates Cu^{1+}, which reacts with BCA to form a complex that absorbs light strongly at 562 nm [6,7]. The absorbance of this complex is then used to quantify an analyte's concentration either using Beer's law ($A = \varepsilon * b * c$) or according to a standard curve.

However, although the BCA assay is robust, a number of compounds interfere with it [8–12]. During the past few decades, a considerable number of compounds that can complicate the quantitation of specific analytes have been identified. The instructions of the most common BCA protein assay kit, produced by a number of manufacturers, acknowledge the potential of false readings from a number of compounds that can interfere with the assay. These include, but are not limited to, mercaptoethanol, phospholipids, hydrogen peroxide, and sulfo-*N*-hydroxysuccinimide, all of which give erroneously high absorption readings [8–12]. Other compounds, such as ammonium sulfate and 2-D Pharmalyte, reduce the amount of color the BCA assay can produce, thereby leading to erroneously low absorption readings [8]. More recently, the assay was used to determine sulfhydryl, *N*-hydroxysuccinimido carboxylate, aldehyde, and hydrazide functional groups on a variety of solid supports [13].

Recently, we found that compounds from a previously unreported structure class, specifically the α-hydroxy ketone structure, influence the accuracy of the BCA assay to give erroneously high absorbance readings. The α-hydroxy ketone class of compounds is susceptible to autoxidation and can enolize via an enediol intermediate during tautomerization [14,15]. These α-hydroxy ketone compounds also can undergo tauromerization in the same manner as reducing sugars [15,16]. Under alkaline conditions, such as those found in the BCA assay (pH 11.25), reducing sugars isomerize to an equilibrium mixture of aldoses and ketoses via the same enediol intermediate [17,18]. However, the α-hydroxy ketone class of compounds is far more effective at reducing the copper than reducing sugars, which causes a very strong absorbance reading in the BCA assay. Therefore, we hypothesized that the ability of α-hydroxy ketones, and by extension reducing sugars, to form an enediol intermediate is the mechanism through which these compounds are amenable to quantitation by the BCA assay. The ability of these chemicals to reduce Cu^{2+} and affect the BCA assay provides a very useful tool in quantifying these compounds. It is also important to note that if an α-hydroxy ketone is present in a sample, it may interfere with the accuracy of the results when conducting the

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[1] *Abbreviations used:* BCA, bicinchoninic acid; DHA, 1,3-dihydroxyacetone; BSA, bovine serum albumin; Na_2CO_3, sodium carbonate; PBS, phosphate-buffered saline; DHAP, dihydroxyacetone phosphate.

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BCA assay on solutions that contain other analytes such as proteins.

Materials and methods

Materials

1,3-Dihydroxyacetone (DHA) dimer, dihydroxyacetone phosphate hemimagnesium salt hydrate, dextrose, fructose, DL-glyceraldehyde dimer, *p*-toluene sulfonic acid, bovine serum albumin (BSA), Bradford reagent, ninhydrin reagent, and 2,2-dihydroxyindane-1,3-dione were purchased from Sigma–Aldrich (St. Louis, MO, USA) and used as received. Acetoin, acetone, ethyl alcohol, ethyl acetate, glycerol, triethyl orthoformate, sodium carbonate (Na_2CO_3), 0.1 N silver nitrate, 28% (w/w) aqueous solution of ammonia, and 0.1 N NaOH were purchased from VWR (West Chester, PA, USA) and used as received. 1,5-Dihydroxypentan-3-one was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used as received. Phosphate-buffered saline (PBS, 10.0 M) without calcium or magnesium was purchased from Bio-Whittaker (Lancaster, MA, USA) and diluted to 1.0 M with 18 Ω water. Corning Costar-brand clear, 96-well cell culture plates were purchased from Fisher (cat. no. 3599). A BCA Protein Assay Kit (product no. 23225) was purchased from Thermo Fisher Scientific.

Synthesis of (2,5-diethoxy-1,4-dioxane-2,5-dimethanol)

The protected DHA dimer was synthesized using a previously reported method [19]. A 500-mg round-bottom flask was charged with DHA dimer (32 g, 177.8 mmol), triethyl orthoformate (60 ml, 360 mmol), and *p*-toluenesulfonic acid (128 mg) in 300 ml of ethyl alcohol. The mixture was stirred for 24 h, after which time 400 mg of Na_2CO_3 was added. The reaction mixture was stirred for an additional 30 min before filtration. The solvent and residual triethyl orthoformate were removed in vacuo, and the product recrystallized from ethyl acetate to yield the title compound (31 g, 74%). ^{1}H NMR ($CDCl_3$) δ: 1.23–1.26 (6H) and 3.53–3.95 (12H). Anal. Calcd: C, 50.85; H, 8.47. Found: C, 51.14; H, 8.70.

BCA assay

Various concentrations, ranging from 1.11×10^{-2} M to 1.72×10^{-4} M, were selected for each compound. All serial dilutions were done with 1× PBS buffer. The assay was run under standard conditions according to the manufacturer's directions, 50:1 BCA and tartrate in an alkaline carbonate buffer (reagent A) to 4% copper sulfate pentahydrate solution (reagent B), and the relative intensity of the reading was determined at 562 nm on a SpectraMax Plus 384 microplate reader (Molecular Devices) after 1 h of incubation at 37 °C.

Copper(II) ion ratios in the BCA assay working reagent

A standard curve for DHA with concentrations ranging from 4.44×10^{-2} M to 6.94×10^{-4} M was created in triplicate. All serial dilutions were done with 1× PBS buffer. The BCA assay solution was then prepared six times, varying the copper concentration by changing the conditions of reagent B while holding the volume of reagent A fixed. The DHA samples were allowed to incubate for 1 h at 37 °C with the various BCA working reagent conditions before the absorbance was read. Due to the saturation of the signal, each sample was diluted 10× postincubation, or 20× in the case of the 4.4×10^{-2} M sample, and the relative percentage absorbance as compared with the largest signal of 4.44×10^{-2} M DHA in a 50:3 working reagent ratio was determined.

Protein sample interference

A standard curve for BSA with six concentrations ranging from 0.5 mg/ml to 1.56×10^{-2} mg/ml and a standard curve for DHA with four concentrations ranging from 0.125 mg/ml to 1.56×10^{-2} mg/ml (1.38×10^{-3} M to 1.72×10^{-4} M) were created in triplicate. All serial dilutions were made with 1× PBS buffer. To each interference trial, the BSA concentration was varied while a constant amount of DHA was added to each sample. This was repeated four times so that each concentration of DHA was added to the entire range of BSA concentrations. The mixed BSA and DHA samples were run in the BCA assay in triplicate using the same conditions listed previously.

Bradford assay

A serial dilution for DHA with concentrations ranging from 1.11×10^{-2} M to 1.72×10^{-4} M was created in triplicate using 1× PBS buffer. The assay was run under the standard conditions as recommended by the supplier's instructions.

Ammoniacal silver nitrate (Tollen's reagent)

In a 20-ml glass vial was charged 150 mg of the α-hydroxy ketone-based analyte in 1 ml of 18 Ω water. To this was added 7.5 ml of 0.1 N silver nitrate and 15 ml of 28% (w/w) solution of aqueous ammonia hydroxide. The vial was then rotated by hand continuously for approximately 5 min at room temperature until a silver mirror or finely divided black precipitate formed.

Ninhydrin protein assay

As per the assay guidelines from Sigma–Aldrich, a 50-μM solution of sample (DHA or glycine) was prepared in 0.05% glacial acetic acid. A 2-ml aliquot was added to a glass test tube, to which 1 ml of ninhydrin reagent (2% solution of ninhydrin and hydrindantin in dimethyl sulfoxide and lithium acetate buffer, pH 5.2) was added. The test tubes were then allowed to sit in boiling water for 10 min before cooling to room temperature and the subsequent addition of 5 ml of 95% ethanol. Then 200 μl of each solution was put in a 96-well plate, and the absorbance was determined at 570 nm.

Ninhydrin enediol test

In a 20-ml vial was charged equimolar amounts (1.0 mol) of 2,2-dihydroxyindane-1,3-dione (ninhydrin) and the α-hydroxy ketone compound DHA or DL-glyceraldehyde. To this was added 10 ml of either 1× PBS, 18 Ω water, 0.1 N NaOH, or reagent A from the BCA assay kit. A 1-ml aliquot was taken from each mixture and placed in a separate tube. To this was added 20 μl of reagent B, and the solution was thoroughly mixed at room temperature for 30 s. The tubes were then centrifuged for 1 min at 3000 rpm to collect any precipitate. The precipitate was then recrystallized in acetone and dried in vacuo. ^{1}H NMR (DMSO-d_6) δ: 6.48 (s; 2H, OH) and 7.97 (s; 8H, aromatic H).

Results and discussion

Role of structural arrangement of the analyte in the BCA assay

To highlight the impact α-hydroxy ketones have on the BCA assay, a variety of compounds either containing the α-hydroxy ketone arrangement or lacking this arrangement were subjected to the BCA assay. Using a consistent molar basis for the study and a