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Measurement of Protein Using Bicinchoninic Acid[1]

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Bicinchoninic acid, sodium salt, is a stable, water-soluble compound capable of forming an intense purple complex with cuprous ion (Cu^{1+}) in an alkaline environment. This reagent forms the basis of an analytical method capable of monitoring cuprous ion produced in the reaction of protein with alkaline Cu^{2+} (biuret reaction). The color produced from this reaction is stable and increases in a proportional fashion over a broad range of increasing protein concentrations. When compared to the method of Lowry *et al.*, the results reported here demonstrate a greater tolerance of the bicinchoninate reagent toward such commonly encountered interferences as nonionic detergents and simple buffer salts. The stability of the reagent and resulting chromophore also allows for a simplified, one-step analysis and an enhanced flexibility in protocol selection. This new method maintains the high sensitivity and low protein-to-protein variation associated with the Lowry technique. © 1985 Academic Press, Inc.

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The widely used method of Lowry *et al.* (1) for protein determination relies on the Folin–Ciocalteau reagent to enhance the sensitivity of the biuret reaction. The instability of this reagent in an alkaline medium demands that exacting technique be exercised in the timing of both reagent addition and mixing with sample in order to obtain accurate results. The two-step nature of the Lowry method is mechanically cumbersome and tedious, and adds considerable complexity to efforts to attempt to utilize it in an automated or post-column-detection format (2). Additionally, nonionic detergents as well as some buffer salts used at levels useful for protein solubilization can interfere by forming insoluble precipitates with the Folin–Ciocalteau reagent. It seems apparent that most of the difficulties associated with the Lowry method are due to the peculiarities of the detection reagent used.

In this paper we describe a new protein measurement method based upon an alternative detection reagent, namely bicinchoninic acid (BCA).[3] In the form of its water-soluble sodium salt, BCA is a sensitive, stable, and highly specific reagent for Cu^{1+} (3). Previously, this attribute has been utilized to monitor the levels of other substances capable of reducing Cu^{2+} to Cu^{1+} such as uric acid (4) and glucose (5). To our knowledge, no one has exploited a BCA indicator system to monitor Cu^{1+} produced during the biuret reaction.

MATERIALS AND METHODS

Chemicals. Bicinchoninic acid, sodium salt, was synthesized by the Pfitzinger reaction of isatin and acetoin (Aldrich), substituting sodium hydroxide for potassium hydroxide in the method of Lesesne and Henze (6). The

[1] This paper is dedicated to the memory of Dr. E. Melvin Gindler, a long-time friend and colleague. Without Mel's knowledge and love of chemistry which he so generously shared, this work would not have been possible.

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[3] Abbreviations used: BCA, bicinchoninic acid; BCA-Na_2, bicinchoninic acid, disodium salt; RIBO, ribonuclease A; CHYMO, chymotrypsinogen; IgG, human immunoglobulin G; BSA, bovine serum albumin; S-WR, Standard Working Reagent.

crude product obtained was subjected to recrystallization from a minimum amount of 75°C water until the absorbance blank of the freshly prepared working reagent (described below) was less than 0.065 at 562 nm. Three recrystallizations were usually required. The hydrated, nearly colorless needles isolated revert to amorphous, cream-colored anhydrous powder upon drying at 60°C. Technical-grade BCA currently available from various sources (Sigma, Hach, Pierce) gives a somewhat higher blank reading and has not been further evaluated. Inorganic salts used in the reagent formulations were of reagent-grade quality (J. T. Baker or Fisher). All prepared solutions were passed through 1-μm filters to remove insoluble debris associated with the salts. Folin–Ciocalteau reagent (2 N) was purchased from Fisher and diluted to 1 N prior to use. Compounds used in the interfering substances studies were of the highest quality commercially available, while nonionic detergents were further purified by described methods (7,8). All water used was deionized, but care must be taken to avoid obtaining water from systems containing copper lines and fittings, because enough dissolved copper as Cu^{1+} may be present to contribute appreciably to blank readings. We found that deionized water (18 Mohm-cm) delivered from all plastic cartridged units (Millipore or Barnsted) was satisfactory.

Proteins. BSA (crystallized) and IgG (human) were obtained from Miles. Ribonuclease, chymotrypsinogen, and human insulin were from Boehringer–Mannheim, and avidin (egg white) was of affinity-purified grade obtained from Pierce. Standard protein solutions were prepared in either isotonic saline or, in the case of the interfering substances studies, in a solution containing the particular compound under study.

Lowry reagent and protocol. The Lowry reagent formulations and protocol used in this study were as described (1).

Standard BCA reagent and protocol. Reagent A consists of an aqueous solution of 1% BCA-Na_2, 2% $Na_2CO_3 \cdot H_2O$, 0.16% Na_2 tartrate, 0.4% NaOH, and 0.95% $NaHCO_3$. If needed, appropriate addition of NaOH (50%) or solid $NaHCO_3$ is made to reagent A to adjust the pH to 11.25. Reagent B consists of 4% $CuSO_4 \cdot 5H_2O$ in deionized water. Reagents A and B are stable indefinitely at room temperature and are commercially available (Pierce). Standard Working Reagent (S-WR) is prepared weekly or as needed by mixing 100 vol of Reagent A with 2 vol of Reagent B. S-WR is apple green in color.

The standard assay procedure consists of mixing 1 vol of sample (standard or unknown) with 20 vol of S-WR in a test tube. For convenience we routinely use a 100-μl sample and 2 ml S-WR; however, any multiple of these volumes may be used depending upon the total volume needs of a particular spectrophotometer or considerations based on the availability of the protein to be assayed. Color development proceeds immediately, even at room temperature, but it can be greatly accelerated by incubating the tubes in a constant-temperature water bath. In this respect, the temperature chosen for the color development is directly related to the desired sensitivity. The incubation protocols used to generate the bulk of the data in this report were (i) room temperature for 2 h, (ii) 37°C for 30 min, and (iii) 60°C for 30 min. After the chosen incubation step, the samples are cooled to room temperature and their absorbances measured at 562 nm versus a reagent blank. The concentration of unknowns can be then determined from a plot of concentration vs absorbances obtained for the standard protein solutions.

pH optimum. The pH optimum of the assay was determined by adjusting the pH of Reagent A with either NaOH (50%) or solid $NaHCO_3$, preparing the corresponding working reagents, and assaying a set of BSA standards (100–1200 μg/ml) using an incubation protocol of 30 min at 37°C.

Color stability. Final color stability was determined for all three selected incubation protocols. After the incubation period, the assay tubes were cooled to room temperature as needed and the absorbances recorded imme-