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Determination of biotin on a protein by quantitative sodium dodecyl sulfate–capillary gel electrophoresis of monomeric avidin

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Abstract

Sodium dodecyl sulfate–capillary gel electrophoresis (SDS–CGE) is performed to quantify monomeric avidin and biotin on a protein. Under non-reducing SDS–CGE conditions, avidin migrates as monomers exhibiting apparent molecular mass 17 000. In the presence of a biotin–protein conjugate, monomeric avidin binds the conjugate and forms a larger complex that migrates later in the separation. The difference between the remaining monomeric avidin and the initial amount is the portion of monomeric avidin bound to the conjugate. Accordingly, the number of biotin on the protein can be calculated. The assay is linearly responsive to increasing biotin loading in a biotinylation reaction of a protein. Accuracy of the assay is also demonstrated by good sample dilution recovery. Excellent quantitative reproducibility <2% (relative standard deviation) is obtained for both intra- and inter-day measurements. Main advantages of the method include the use of monomeric avidin that minimizes steric hindrance to capture biotin on a protein and assay automation on a capillary electrophoresis apparatus.

Keywords: Biotin; Proteins; Avidin; Glycoproteins

1. Introduction

Avidin is a biotin binding glycoprotein [1]. It is composed of four active subunits. The strong interaction (K_D~10^{-15}) [2] between avidin and biotin makes them widely used in many commercial assay formats and systems. In the diagnostics industry, proteins of interest are biotinylated to bind firmly to avidin on the particles or in solution. Accurate and precise determination for the biotin-to-protein molar incorporation ratio is therefore important to ensure the quality and proper performance of a biotinylated protein conjugate in a given assay system.

Although there have been numerous analytical techniques and methods reported for measuring biotin [3], perhaps the simplest has been the spectrophotometric method using the dye HABA (4-hydroxyazobenzene-2′-carboxylic acid) [4]. The assay is based on spectral change or shift of the HABA dye at 500 nm when bound to avidin. Biotin, due to its high binding affinity to avidin, can displace HABA and be then indirectly quantified by measuring the spectral change. However, when biotin is attached to a protein, the HABA method becomes less effective for two obvious reasons: steric hindrance and decreased affinity of the biotin to avidin. Similarly, many other biotin binding assays (homogeneous and heterogeneous) involving various labels and separation means have used specific binding protein

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avidin or structurally similar streptavdin [3,5] and would, to some extent, encounter the above limitations.

Sodium dodecyl sulfate–capillary gel electrophoresis (SDS–CGE) is a high-performance version of the conventional SDS–polyacrylamide gel electrophoresis (PAGE). Under the SDS conditions, we in this study, and Hiller et al. [6] using SDS–PAGE have found that avidin separates as monomers exhibiting apparent molecular mass (M_r) 17 000 vs. the expected size of a tetramer (M_r 67 000). This observation plus the quantitative advantages associated with SDS–CGE [7] make the capillary technique highly suitable for quantitative analysis of the monomeric avidin (m-avidin). Using an appropriate internal protein reference that migrates near the analyte of interest, excellent quantitative precision (RSD<2%) can be achieved.

Under the non-reducing SDS–CGE conditions, binding between m-avidin and biotin protein conjugate occurs and forms a larger protein complex that migrates later in the separation. Therefore, by simply quantifying the peak(s) representing the m-avidin before and after the binding on SDS–CGE, the amount of m-avidin reacted can be determined and used to calculate moles of biotin in a protein conjugate. This approach also minimizes steric hindrance by using m-avidin as a smaller specific binding protein vs. the complete avidin molecule and is more accurate. A simple stoichiometric relationship (1:1, m-avidin:biotin) exists for the binding and for the calculation. The size of a biotin protein conjugate does not affect the method so long that the conjugate/m-avidin complex(es) migrate at a location that does not interfere with the quantification of the m-avidin peak(s). Furthermore, the assay is conveniently automated on a capillary electrophoretic apparatus.

2. Experimental

2.1. Reagent and materials

A CE-SDS protein kit (Bio-Rad, Hercules, CA, USA) was used. Sodium phosphate, sodium chloride, hydrochloric acid (1 *M*) and sodium hydroxide (1 *M*) were from Fisher (Fair Lawn, NJ, USA). Fused-silica capillaries of 75 μm I.D.×360 μm O.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). Biotinylated protein conjugates ''X, Y, and Z'' were prepared and purified at Bayer, Diagnostics Division (Tarrytown, NY, USA). Murine monoclonal antibody was also affinity purified at Bayer, Diagnostic Division. Horse heart cytochrome *c* (cyt c) and egg white avidin were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was from Pentex Division (Bayer, Kankakee, IL, USA).

2.2. Instrumentation

SDS–CGE was performed on an Agilent Technologies ^{3D}CE CHEMSTATION (Waldbronn, Germany) equipped with a Hewlett-Packard Vectra VE pentium II computer. Signals were detected at 220 nm using the electropherograph's built-in diode array detection system. Temperature was regulated to 20 °C by the instrument's air-circulating heating and cooling system. Operation of the instrument and data collection/analysis were controlled by Agilent Technologies CHEMSTATION system software revision A.06.03 (509).

2.3. Method

2.3.1. Sample preparation

The avidin solution is prepared as 10× stock of the following components: SDS–sample buffer from the kit (20 μl), cyt c (5 μl at 1.5 mg/ml), and avidin (5 μl at 5.0 mg/ml). Cytochrome *c* (M_r~12 000) migrates immediately before m-avidin and is used as internal reference for quantitative analysis of m-avidin. The stock solution (30 μl) is mixed with 10 μl of sample or water. The final mixture is then heated at 70 °C for 5 min on a Reacti-Therm III heating module (Pierce, Rockford, IL, USA).

2.3.2. SDS–CGE

SDS–CGE was performed according to the previously described procedure [8]. The Agilent Technologies CHEMSTATION software corrects the peak area for migration time. In the text, the term ''peak area ratio'' equals to corrected peak area (m-avidin) divided by corrected peak area (internal reference, cty c).

Calculation

$$\text{mole (m-avidin reacted)} = \frac{(A - B) \times C \times D}{E} \quad (1)$$

where A is the initial avidin amount (μg); B the avidin remained (μg); C the number of monomers per avidin (4); D the conversion factor from μg to g (10^{-6}); and E the M_r of avidin (67 500).

$$\text{mole (m-avidin reacted)} = (A - B) \times 5.926 \times 10^{-11}\ \text{mole}/\mu\text{g} \quad (2)$$

3. Results and discussion

Fig. 1 shows an electropherogram of a protein mixture [cyt c (peak 1), m-avidin (peak 2), BSA (peak 3), and a murine monoclonal antibody (peak 4)]. According to the migration time of the ''avidin'' in the electropherogram, it has clearly broken down into monomers under the SDS–CGE conditions. (Note that tetrameric avidin ought to migrate near BSA but not cyt c). Hiller et al. [6] have also previously reported similar observation using SDS–PAGE and indicated that the distinct peaks for the protein represent the glycoforms. Glycosylation does not affect avidin/biotin interaction. These glycoforms are also apparent in the SDS–CGE separation (see also Figs. 2 and 3 below).

This study applies the SDS–CGE separation of m-avidin to the titration of biotin on a protein. If a biotinylated protein is allowed to pre-mix with m-avidin in the SDS–sample buffer, binding occurs between m-avidin and the biotin on the protein. Fig. 2 shows the electrophoretic behavior. Electropherogram A in the figure shows the separation of a biotinylated murine monoclonal antibody (conjugate ''X'') in the absence of any m-avidin. In electropherogram B, a known amount of m-avidin is separated. Mixing the biotinylated antibody and m-avidin creates ''m-avidin + antibody'' complexes that migrate later (electropherogram C). At the same time, peak-area of the m-avidin (peak 2) is reduced. Other than to facilitate the breakdown of tetrameric avidin to monomers, the extent that SDS (0.1%) in the replaceable polymer solution has effect on the binding affinity of avidin is unknown. However, it is apparent that the high binding affinity of avidin is not significantly affected by the SDS–CGE conditions such that the ''gel-shift'' phenomenon would not have completely occurred. In many instances where biotin-to-protein ratios are greater than 1 (e.g. electropherogram C, Fig. 2), none of the biotinylated proteins can be detected without binding to m-avidin. Therefore, the influence, if any, of the SDS at 0.1% on the proposed system is most likely insignificant. Under the same experimental conditions, we have observed that the ''gel-shift'' phenomenon could work even on an antibody–antigen system [8]. In a related study, Nakamura et al. used an avidin–

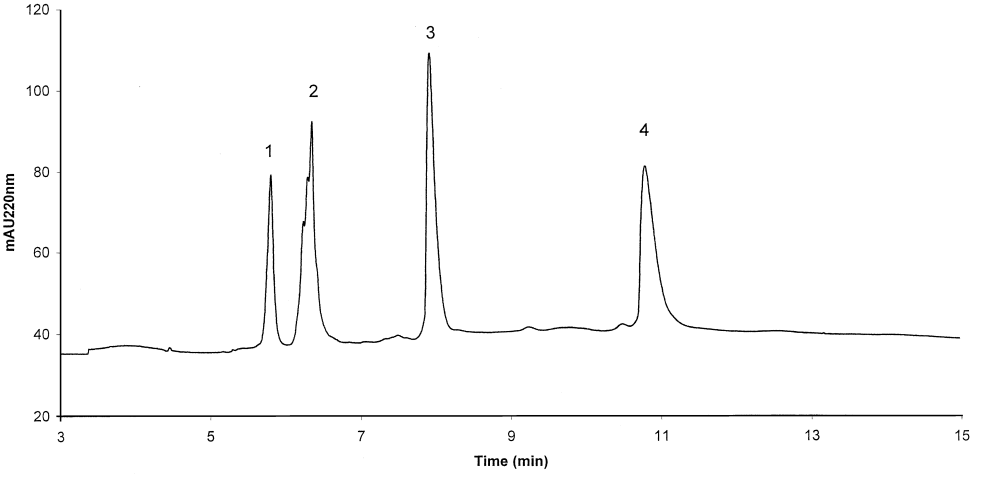

Fig. 1. Electropherogram of a protein mixture. Peaks represent cyt c (1), m-avidin (2), BSA (3), and murine monoclonal antibody (4).

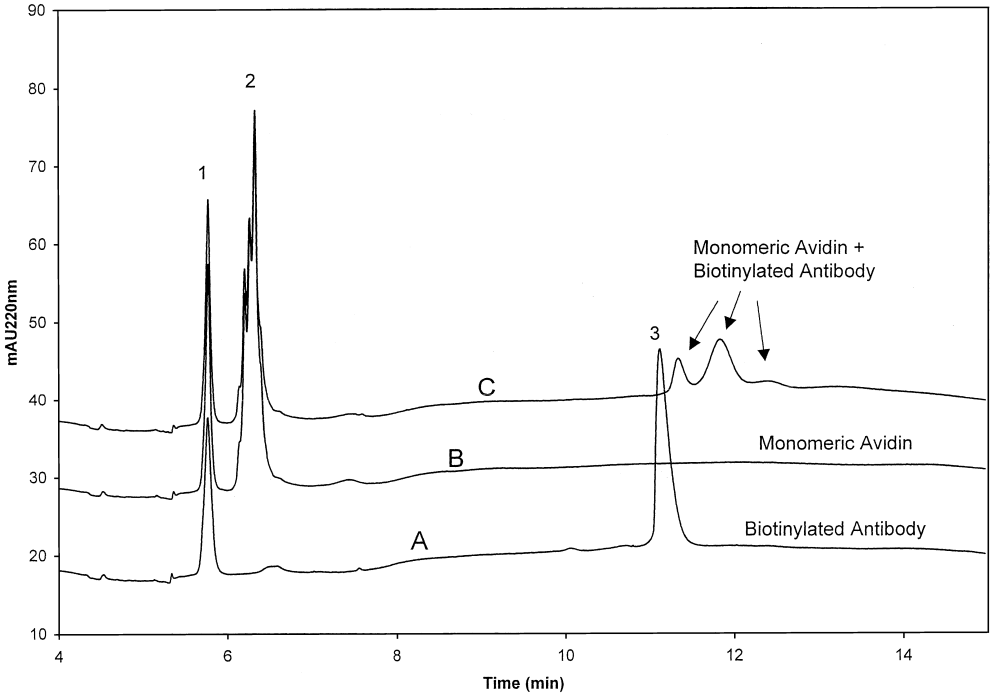

Fig. 2. Electropherograms of a biotinylated monoclonal antibody (b-Ab, peak 3) (A), m-avidin (peak 2) (B), and b-Ab + m-avidin (C). Peak 1 = cty c, and the ''b-Ab + m-avidin'' complexes are seen in electropherogram C.

fluorescein conjugate to directly detect and quantify biotinylated proteins on SDS–PAGE gels [10].

The results in Fig. 2 present an opportunity to quantify biotin on a protein based on the ''gel-shift'' phenomenon and to use m-avidin as a binding indicator. Provided that the m-avidin peaks may be precisely quantified, direct titration of biotin on a protein can be performed. The only other requirement is that the ''m-avidin + biotinylated protein'' complexes do not interfere with the quantification of the m-avidin peaks. In most cases, this requirement is easily met.

In Fig. 3, m-avidin at various concentrations (5.0, 2.5, 1.3, and 0.6 mg/ml) was separated. Each of the three major glycoforms (peaks 1–3) could be quantified and expressed as peak-area ratio (peak-area under the peak(s)/peak-area of the internal reference). The use of a protein internal reference has been shown to greatly enhance quantitative reproducibility on SDS–CGE [7]. The inset in Fig. 3 shows the different plots (peak-area ratio vs. μg-avidin). Although the calibration curve using only peak 1 or peaks 1 + 2 is less steep than that using peaks 1–3, they all show excellent linearity with correlation coefficients ($R^2 \geq 0.999$). Because these different m-avidin peaks represent differences in glycosylation and because carbohydrate content on avidin does not affect its ability to bind biotin [6], each of the three calibration curves may be used. Total peak area under the m-avidin peaks 1–3 is most conveniently used. However, in rare circumstances when the analyte protein (with or without biotin) interfere with a portion of the m-avidin peaks, selected use of peak 1 only, for instance, may be necessary.

Reproducibility for quantitative analysis of m-avidin is shown in Table 1. The control uses water as a sample and represents total m-avidin input. Conjugate ''Y'' is a biotinylated protein. As expected, the m-avidin/cyt c ratio (1.374 ± 0.016) is significantly lower than the control (1.874 ± 0.031). Data ($n = 6$) represent two separate sample preparations and the use of a new capillary on two different days. RSD values are 1.68 and 1.15% for the control and the

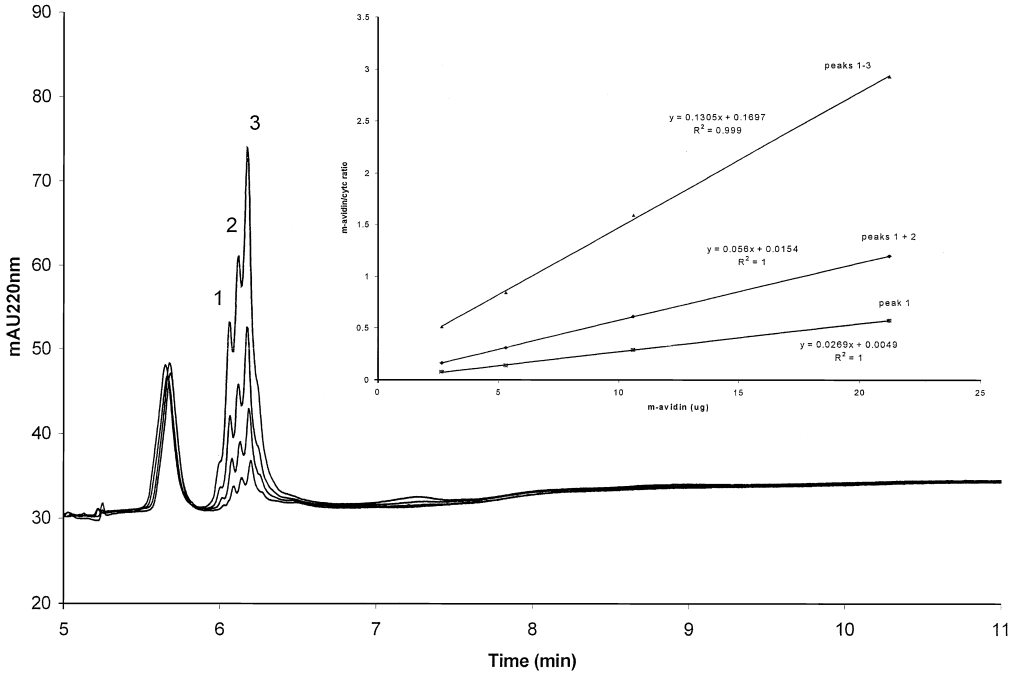

Fig. 3. Electropherograms of m-avidin at various concentrations (5.0, 2.5, 1.3, and 0.63 mg/ml). In this figure, peaks 1–3 represent major glycoforms of the m-avidin. The inset contains calibration curves (peak-area ratio vs. m-avidin (μg)) from peak 1 only, peaks 1+2, and peaks 1–3. Cty c is present as an internal reference.

conjugate sample, respectively. The table demonstrates that quantitative precision for the peak-area ratio determination is excellent (RSD <2%) and that the use of the internal reference protein has eliminated many factors that might adversely affect quantitative precision on SDS–CGE and on a capillary electrophoresis apparatus in general [7,9].

The linear relationship between increases in the determined ratio and in the biotin loading ratio in a biotinylation reaction is evaluated using a biotin-protein conjugate ''Z'' prepared at different biotin loading ratios (5×, 10×, 15×, and 20×). The biotin-to-protein ratios determined by the method are 0.7, 1.1, 1.4, and 1.8, respectively. The linear relationship (the determined incorporation ratio vs. biotin loading ratio) is excellent ($R^2 = 0.997$, equation: $y = 0.072x + 0.35$). Another way to examine the data is to observe the actual increase vs. the theoretical increase. In this instance, biotin loading is doubled from 5× to 10× and also from 10× to 20×. The actual increases determined are 79 and 82%, respectively, of the theoretical maximum increases.

Assay accuracy also was evaluated using the

Table 1
Reproducibility of the m-avidin/cyt c ratio determination

Sample	m-avidin/cyt c ratio[a]						Average	SD	RSD (%)
	Injection number								
	1	2	3	4	5	6			
Water	1.896	1.890	1.848	1.918	1.839	1.856	1.874	0.031	1.68
Conjugate ''Y''	1.387	1.352	1.375	1.380	1.358	1.391	1.374	0.016	1.15

[a] Data represent two separate sample preparations and the use of a new capillary on 2 different days.

Table 2
Biotin-to-protein ratios of conjugate ''X'' determined at different dilutions and at different m-avidin concentrations

Experiment[a]	Dilution	m-avidin/cyt c ratio[b]	% of the total ratio[c]	Biotin-to-protein ratio
A	1×	0.783	52	2.29
B	1×	1.41	63	2.82
B	2-fold	1.84	82	2.71
B	4-fold	2.03	91	2.82

[a] Experiment A uses less (ca. 40%) m-avidin than Experiment B.
[b] Value represents average of two determinations.
[c] ''% of the total ratio'' equals [(m-avidin/cyt c ratio for a sample)/(m-avidin/cyt c ratio for control)]×100.

biotin conjugate ''X'' at various dilutions and at different m-avidin concentrations (Table 2). In the table, the conjugate at 1× concentration was first incubated with two different amounts of m-avidin. In experiment A, m-avidin input was less (by ca. 40%) than experiment B. In B, the conjugate was further diluted 2-fold and 4-fold but same amount of the m-avidin was used. The biotin-to-protein ratio [2.78 (average)±0.06 (SD)] determined was well within acceptable precision and accuracy in experiment B. The ratio (2.29) determined in experiment A was significantly lower. This discrepancy was unexpected but led us to focus on the ''% of the total'' column in the table. Greater percentage means more m-avidin present in the reaction mixture. Green and Toms [11] studied subunits of avidin on agarose beads and found three different classes. One class (ca. 40% of the subunits) had binding affinity equaled that of a tetrameric avidin. One third had reduced affinity ($K_D \sim 10^{-10}$ M), and 25% had even weaker affinity ($K_D = 5\times10^{-8}$ M). Based on the information, experiment A (52% of the total m-avidin/cyt c ratio) used part of the second class of the subunits. Accordingly, binding apparently became less effective and lower biotin-to-protein ratio was determined. It is therefore recommended that the experimental m-avidin/cyt c ratio ought to be ≥60% of the control (or total m-avidin input ratio) to maximize accuracy of the method.

Finally, Table 3 shows that the method is highly reproducible. Biotin-to-protein ratio was determined for conjugage ''Z'' on 4 different days ($n=8$ total). On each day, fresh avidin reagent mixture (also containing SDS–sample buffer and cyt c) was prepared and used. Day 4 used fresh avidin stock solution. Determination was performed in duplicate daily. Excellent inter-day reproducibility (RSD< 2%) was obtained.

4. Conclusion

Quantitative SDS–CGE of m-avidin is an excellent method for determining biotin incorporation on a protein. Fig. 1 clearly shows the breakdown of avidin into individual subunits under the SDS–CGE conditions. Due to the strong affinity, m-avidin binds biotin on a biotinylated protein under non-reducing SDS conditions. The m-avidin/biotinylated protein complexes then migrate later as larger components. The use of internal reference protein cyt c ensures reproducibility for quantitative analysis of the m-avidin peak(s). Proportional increase of the determined biotin-to-protein ratio when biotin loading

Table 3
Reproducibility of the biotin-to-protein ratio determination for conjugate ''Z''

Biotin-to-protein ratio[a]										
Day 1		Day 2		Day 3		Day 4[b]		Average	SD	RSD (%)
No. 1	No. 2	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2			
2.97	2.95	3.06	2.93	2.98	3.02	2.98	2.97	2.98	0.04	1.36

[a] On each day, fresh avidin working reagent was prepared and used.
[b] On day 4, a fresh avidin stock solution was prepared and used.

ratio is increased in a biotinylation reaction demonstrates the method's accuracy. Data in the dilution recovery experiment (Table 2) indicate that m-avidin in the assay must be present at $\geq$60% more than biotin to further ensure accuracy. The method also gives excellent reproducibility (RSD$<$2%) for both intra- and inter-day measurements. It represents an improved method to ensure consistency in biotin labeling of a protein and to determine the labeling ratio.

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