WHITE PAPER

An Accurate and Simple Spectrophotometric Assay System for Quantitation of Biotin:

The QuantTag™ Biotin Kit

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The binding of biotin to avidin is utilized in a wide variety of detection methodologies. Quantitation of biotin is important for optimization of biotinylation procedures. This study shows that the QuantTag Biotin Kit is more accurate and simpler than the traditional avidin-based HABA assay.

Introduction

The virtually irreversible binding of the vitamin biotin to avidin and streptavidin is the basis for the vast array of detection schemes that have become routine in biotechnology. Biotin labeling of detection reagents is typically accomplished through the use of various reactive forms of biotin, such as biotin N-hydroxysuccinimide ester (biotin-NHS), biotin hydrazide, and others. The degree of labeling can be critical for downstream applications. Low biotin density may result in reduced interaction with the (strept)avidin component whereas over labeling can reduce activity of the labeled molecule (1). Quantitation of biotin labels can aid in optimization of biotinylation protocols.

Biotin is not directly detectable by standard UV or visible spectrophotometry. For many years, the standard method for determining biotinylation has been the spectrophotometric 2-(4'-hydroxyazobenzene) benzoic acid (HABA) assay developed by Green (2). The dye HABA binds avidin with low affinity. Upon binding of biotin, the HABA is displaced causing a measurable shift of the absorbance maxima of the dye. The decrease of absorbance at 500 nm for a biotinylated sample is compared to that of a set of biotin standards in order to determine the number of biotins in the sample.

Avidin-based assays, including the HABA assay, suffer from inaccuracies caused by steric hindrance that prevent closely spaced biotins or biotins "buried" within secondary structure from accessing a binding site on avidin. To reduce this problem, samples must be pre-treated with hydrolytic enzymes such as Pronase for proteins or DNase I, for nucleic acids. Unfortunately, enzymatic degradation requires several hours with no certainty that complete digestion is attained or that steric problems are eliminated.

In recent years, mass spectrometry (MS) has been shown to be an accurate method for biotin determination on proteins (3,4). The number of biotins is determined by comparison of the protein's mass before and after biotinylation. Unlike avidin-based methods, mass spectrometry does not suffer from steric hindrance and samples do not require degradation. However, obtaining useful data is difficult for proteins larger than about 60 κD. Also, the cost of assaying a large number of samples may be prohibitive.

An excellent alternative to (strept)avidin-based assays or MS is the QuantTag Biotin Kit from Vector Laboratories. The QuantTag Kit contains proprietary, low molecular weight chemical reagents which react with biotin, yielding a visible color change which is quantified spectrophotometrically. Because steric interference is not an issue, accurate biotin determination is possible without digestion, saving valuable time and eliminating the introduction of assay variability. The QuantTag Kit has been used to quantify biotins on proteins (5-7), peptides (8), heparin (9-12), and lipopolysaccharides (13).

In this article, it will be shown that the QuantTag assay is more accurate than the HABA assay, even with sample digestion.

Materials and Methods

HABA and Pronase (type XIV from *Streptomyces griseus*) were obtained from Sigma. Biotin-labeled proteins and Biotin (LongArm) N-hydroxysuccinamide ester (biotin-NHS) were from Vector Laboratories.

Biotin-labeled and unlabeled Protein A were analyzed by electrospray ionization MS by the Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University Mass Spectrometry. Data was provided deconvoluted and plotted as mass vs. intensity.

Bovine serum albumin (BSA) biotinylation: BSA was dissolved at 6 mg/ml in 10 mm HEPES, 150 mm NaCl, pH 8.5. Varying volumes of 50 mg/ml biotin-NHS (Vector Laboratories) in DMSO was added and the mixture incubated at 25°C. After 2 hours, all reactions were quenched with glycine. Unincorporated biotin reagent was removed by extensive filtration using a Sartorius Stedim Biotech VivaSpin® 500 centrifugal filter.

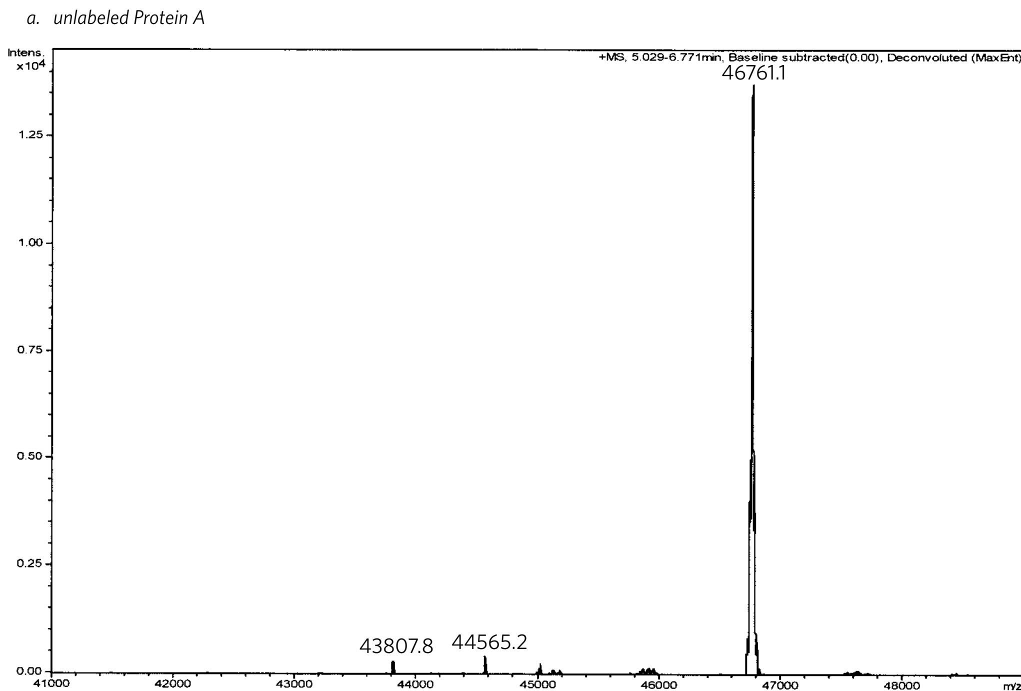
Digestion of biotinylated protein: 50 ul of 10 mg/ml protein in 10 mm HEPES, 150 mm NaCl, pH 7.4 was heated to 56°C for 10 min. Pronase was added to a concentration of 0.1 % and the mixture incubated overnight at 25°C.

HABA assay: For each sample, 100 ul of 0.5 mg/ml avidin in 50 mm sodium phosphate, 150 mm NaCl, pH 6.0 was mixed with 2.5 µl of 10 mm HABA in 10 mm NaOH and 1 µl of 10 mg/ml biotin-labeled protein or 1 µl of various biotin standard solutions. Samples were incubated for 5 min at 25°C. After the spectrophotometer was "zeroed" on an empty cuvette, absorbance of samples and standards was measured at 500 nm. A standard curve of the biotin standards was prepared and the biotinylated proteins compared to the curve.

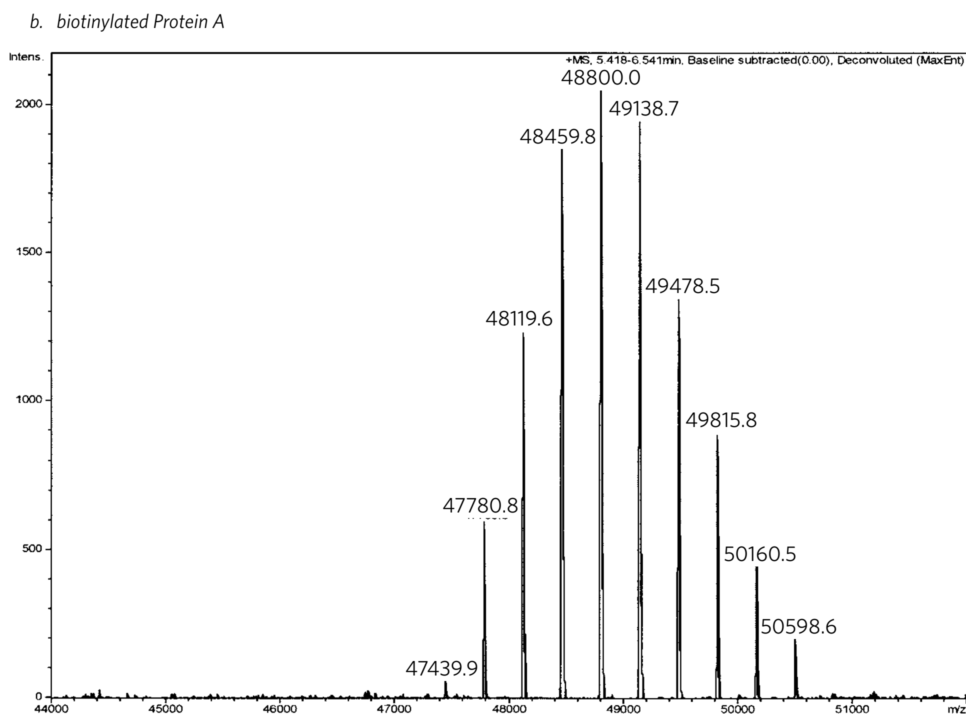
QuantTag assay: The QuantTag assay was performed according to the protocol provided with the kit. Briefly, a working solution was prepared by mixing equal parts of Reagent 1 and Reagent 2 with 0.1 volumes of Reagent 3. One ml of working solution was added to 20 µl of biotinylated protein solution or various volumes of biotin standard solution (provided in the kit). After incubation at room temperature for 30 min, absorbance was measured at 535 nm in a spectrophotometer. A standard curve was prepared of biotin standards and biotinylated protein samples were compared to the curve.

Figure 1. Mass spectrometry of Protein A

a. unlabeled Protein A



b. biotinylated Protein A



Results and Discussion

To compare different analytical methods, biotin-labeled and unlabeled Protein A were analyzed by MS, HABA, and QuantTag. Figure 1a shows that unmodified Protein A is a single species with a mass of 46,761 g/mol. Figure 1b shows biotin-labeled Protein A. The expected increase in mass contributed by a single biotin label would be 338.2 g/mol. The spectrum demonstrates an increase in mass, following a normal distribution, corresponding to about 2 to 11 biotins with an average of about 6 biotins.

Table 1. *Analysis of biotin-labeled Protein A by MS, QuantTag, and HABA*

	Ave. mols biotin/mol Protein A
MS	6
QuantTag	5.89 ± 0.18
HABA (undigested)	2.24 ± 0.13
HABA (digested)	2.28 ± 0.12

The same biotinylated Protein A sample was tested using the QuantTag Biotin Kit and the HABA assay. Table 1 shows that QuantTag gives a result very much in agreement with MS. However, HABA, even after overnight enzymatic digestion, significantly underestimates the number of biotins.

Because of the inaccuracy of the HABA assay compared to the QuantTag assay shown above, and the apparent indifference of the HABA assay to sample degradation, it was decided to see if this situation was unique to Protein A. A variety of biotinylated proteins were analyzed by the QuantTag and HABA assays. Table 2 shows that in some, but not all, cases digestion of the sample improved the accuracy of the HABA assay. However, in all measurable cases HABA underestimated the degree of biotinylation.

Also, upon addition of the HABA/avidin solution to wheat germ agglutinin and alkaline phosphatase, the proteins precipitated. Precipitation of the samples caused light scattering that interfered with spectrophotometric measurement of the sample.

Table 2. *QuantTag vs. HABA*

	QuantTag	HABA (undigested)	HABA (digested)
Horse IgG	30.92 ± 0.72	11.525 ± 3.18	15.25 ± 1.14
Goat IgG	34.77 ± 0.23	14.59 4 ± 1.43	19.75 ± 0.75
Horseradish peroxidase	3.91 ± 0.15	2.17 ± 1.32	0.44 ± 0.21
Lycopersicon esculentum lectin	16.19 ± 2.51	7.36 ± 0.94	7.17 ± 0.73
Aleuria aurantia lectin	12.40 ± 0.82	2.28 ± 1.52	4.38 ± 0.19
Wheat germ agglutinin	6.69 ± 0.36	precipitated	precipitated
Alkaline phosphatase	10.19 ± 1.98	precipitated	precipitated

This precipitation is indicative of macromolecular complex formation due to crosslinking of avidin through proteins containing multiple biotins. Crosslinking by this mechanism in the digested samples shows that enzymatic digestion does not always achieve the desired goal.

Samples that precipitated were not the most densely labeled. In order to determine the effect of the degree of biotinylation on each assay, bovine serum albumin (BSA) was labeled with various amounts of biotinylation reagent. After purification, the labled BSA samples were analyzed by QuantTag and HABA assays. The data in Table 3 shows that digestion of the BSA improved the HABA assay results. Additionally, digestion prevented precipitation of the more heavily labeled HABA samples, probably by cleavage of the peptide bonds connecting biotins. However, at all levels of biotinylation, HABA underestimated the number of biotins per protein.

Table 3. *Effects of biotin density*

Molar excess of Biotin-NHS over BSA	QuantTag	HABA (undigested)	HABA (digested)
3.7	3.88 ± 0.05	0.71 ± 0.06	1.27 ± 0.19
7.3	6.84 ± 0.34	1.61 ± 0.03	2.40 ± 0.18
14.8	12.12 ± 0.54	2.91 ± 0.11	4.14 ± 0.65
29.6	24.53 ± 0.78	precipitated	9.96 ± 0.33
59.0	45.75 ± 1.48	precipitated	14.67 ± 0.01

Conclusion

Quantification of biotins on detection reagents is critical for optimization of the biotinylation reaction. The HABA assay, the traditional method for biotin determination, suffers from inaccuracy caused by steric hindrance. Commercial biotin quantitation kits based on avidin binding share the same shortfalls as the HABA assay. Enzymatic digestion of biotinylated samples may reduce, but does not eliminate, the steric problems. MS offers an accurate alternative to avidin-based methods.

However, protein size, heterogeneity, and cost are all limiting factors in MS. In this article, it has be shown that the QuantTag Biotin Kit offers a simple and accurate method for biotin quantitation with no sample digestion required.

Conclusion

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