ELSEVIER

Journal of Nutritional Biochemistry 14 (2003) 196-202

THE JOURNAL OF Nutritional Biochemistry

Detection and quantification of biotinylated proteins using the Storm 840 Optical Scanner

Brandon Lewis, Sara Rathman, Robert J. McMahon*

Food Science and Human Nutrition Department, Institute of Food and Agricultural Sciences and the, College of Agricultural and Life Sciences, University of Florida, Gainesville, FL 32611-0370

Received 11 June 2002; received in revised form 13 November 2002; accepted 8 December 2002

Abstract

The use of the avidin-biotin interaction is becoming an increasingly common method for the detection of proteins. The use of fluorescence detection with avidin-biotin systems has the potential to greatly increase both the sensitivity and linearity of this type of analysis. In this report, three fluorescent systems were tested for their ability to detect biotinylated polypeptides in purified and complex biological samples. These systems include a Neutravidin-Alexa Fluor430 conjugate, an avidin–horseradish peroxidase conjugate with the ECL-Plus detection system, and an avidin–alkaline phosphatase conjugate with the ECF detection system. Biotinylated molecular weight standards, biotinylated bovine serum albumin, and rat liver homogenate were resolved by SDS-PAGE gel electrophoresis and transferred to polyvinyldifluoride membrane. Biotinylated polypeptides were then visualized on the Storm840 optical scanner. The Neutravidin–Alexa Fluor430 conjugate exhibited the lowest sensitivity, but displayed high linearity. The avidin–horseradish peroxidase and avidin–alkaline phosphatase conjugates, when combined with appropriate fluorescent substrates, exhibited much higher fluorescence, with the avidin–alkaline phosphatase ECF system displaying the highest sensitivity. All systems demonstrated an ability to reliably detect and quantify biotinylated polypeptides in purified as well as complex samples, given careful attention to conditions optimized for each system.

Keywords: Avidin blotting; Biotinylation; ECL-Plus; ECF; Storm840; Alexa Fluor430

1. Introduction

The very low dissociation constant of the avidin–biotin interaction (10^{-15} M^{-1}) has been exploited in a variety of biotechnology applications. These uses include receptor characterization, microbial adhesion, ligand binding, and intracellular trafficking [1–4]. Biotin analogs with reactive chemistries towards several types of organic molecules are also available. For example, the N-hydroxysuccinimide ester of biotin (NHS-biotin) is reactive towards primary amines, typically found on lysine residues and the amino terminus of polypeptides. Other biotin analogs are reactive towards sulfhydryls, oxidized carbohydrates, carboxyl groups, and nucleic acids, while membrane impermeant biotin analogs allow for selective cell surface protein labeling [5]. Conjugation of biotin by this approach allows for the incorporation of a highly specific tag that usually does not disturb the function of the target protein or molecule [6]. Avidin, in an enzymatically or alternatively labeled derivatized form, can then be used to detect and localize biotinylated target molecules.

Ideally, a system used to analyze biotinylated proteins should be both specific and predictable, and the sensitivity of such a system should be compatible with the concentration of biotinylated proteins commonly found in purified or complex biological samples. Several systems for the detection of biotinylated proteins have been established, each with its own advantages and disadvantages [7–13]. The advent of instrumentation capable of detecting and subsequently quantifying fluorescent molecules could potentially lead to greater sensitivity and accuracy in the analysis of biotinylated target polypeptides. One such instrument is the Storm 840 from Molecular Dynamics, which is an optical scanner that performs a dual function of detecting fluorescence from storage phosphor screens or inherently fluorescent samples. The Storm840 scanner excites fluorescent

* Corresponding author. Tel.: +1-812-429-7720; fax: +1-812-429-5904.

E-mail address: robert.j.mcmahon@bms.com (R.J. McMahon).

0955-2863/03/$ – see front matter
doi:10.1016/S0955-2863(02)00283-8

samples with a beam of light at 450 nm and detects light emitted at a wavelength of 520 nm or longer. Several approaches for the production of fluorescent molecules with these parameters have been described, but the utility of quantifying biotinylated proteins using this system has not been previously investigated.

In this report, we describe the assessment of three methods of detecting and quantifying biotinylated polypeptides in biological samples, both in terms of sensitivity and dynamic range. The first system is an avidin–alkaline phosphatase enzyme conjugate and enzymatic generation of a fluorescent product. This system, under the commercial name of ECF western blotting substrate, exhibits a maximum excitation wavelength of 440 nm and a maximum emission of 560 nm. The second system utilizes Neutravidin that has been covalently labeled with the fluorescent dye Alexa Fluor430, which exhibits a maximum excitation wavelength of 430 nm and a maximum emission wavelength of 540 nm. The third system utilizes an avidin - horseradish peroxidase conjugate and the ECL-Plus substrate, whose resulting chemiluminescent product also exhibits fluorescent properties with a maximum excitation wavelength of approximately 420 nm and a maximum emission wavelength of approximately 460 nm. We demonstrate in this report that given careful attention to the appropriate set of conditions and corresponding limitations, each system can be used to accurately assess changes in the abundance of biotinylated polypeptides in both purified samples as well as complex mixtures. This information should lead to a greater exploitation of the Storm840 that is becoming more common in modern life science laboratories.

2. Materials and methods

2.1. Reagents

Neutravidin, a deglycosylated and isoelectrically neutral form of avidin, Neutravidin-horseradish peroxidase, and Neutravidin-alkaline phosphatase were purchased from Pierce (Rockford, IL). Neutravidin was conjugated to Alexa Fluor430 as directed by the manufacturer (Molecular Probes Eugene, OR). Size exclusion columns (DG-10) and biotinylated protein standards were purchased from Bio-Rad Laboratories (Hercules, CA). A protease inhibitor cocktail, containing 4-(2-aminoethyl)-benzenesulfonyl-L-leucyclamido (4-guanidino) butane (E-64), bestatin, leupeptin, and aprotinin, was obtained from Sigma (St. Louis, MO). The Storm 840 Optical Scanner System and ImageQuant Solutions for Windows NT software were from Molecular Dynamics (Amersham Pharmacia Biotech, Piscataway, NJ). All solutions were prepared using highly purified 18 MΩ water (Milli-Q Water Purification System, Millipore, Bedford, MA). The following buffers were used: 100 mmol/L sodium bicarbonate, pH 8.3; HEM buffer [300 mmol/L mannitol, 10 mmol/L sodium N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonate) (HEPES) pH 7.4, 1 mmol/L N,N′-1,2-Ethanediyl-bis[N-(carboxymethyl)glycine] Ethylenediaminetetracetic acid (EDTA), 1:100 (v/v) dilution of Protease Inhibitor Cocktail]; phosphate buffered saline [PBS, 10 mmol/L sodium phosphate, pH 7.2, 150 mmol/L NaCl]; and Tris-buffered saline [TBS-T, 20 mmol/L Tris, pH 7.2, 150 mmol/L NaCl, 0.05% (v/v) Tween–20].

2.2. Synthesis of biotinylated bovine serum albumin

500 mg fatty acid-free bovine serum albumin (fafBSA) was dissolved in ice-cold 0.1 mol/L $NaHCO_3$, pH 7.5 to yield a final concentration of 10 mg/ml. 60 mg N-hydroxysuccinimide biotin ester (NHS-biotin) was dissolved in 5 ml DMSO to yield a final concentration of 12 mg/ml. 5 ml of the NHS-biotin were added to 50 ml of the fafBSA solution and incubated overnight at 4°C with gentle stirring. The solution was then dialyzed (30,000 daltons nominal molecular weight cutoff) in several changes of PBS at 4°C over 48 h. The biotinylation ratio of this preparation was determined through displacement of HABA as previously described [14]. Briefly, HABA is incubated in the presence of avidin which results in a steady state absorption maximum at 500 nm. Biotin containing sample is then added, displacing the HABA dye as the avidin has higher affinity for the biotin than the dye. This displacement reduces the absorption at 500 nm, which is directly related to the concentration of biotin in the sample. However, in the case of biotinylated proteins this method provides only an estimate of the extent of biotinylation since some of the protein bound biotin molecules may be sterically hindered from interacting with the avidin.

2.3. Preparation of liver extracts

A male Sprague Dawley rat, 100 g, was housed individually in a stainless steel hanging cage and maintained on normal rat chow and municipal water. The rat was then anesthetized under halothane vapor and killed by exsanguination. Approximately 300 mg of liver was removed and homogenized in ten volumes HEM buffer. An aliquot of homogenate was removed and quickly frozen in a dry ice/isopropanol bath and stored at −80°C until analyzed for total protein content. The protein concentration of each sample was determined as previously described [15]. The University of Florida Institutional Animal Care and Use Committee approved all procedures.

2.4. Electrophoresis and electroblotting

Biotinylated protein standards, biotinylated bovine serum album, and liver proteins were resolved electrophoretically and transferred to polyvinyldifluoride membrane (PVDF) as previously described [16]. After transfer, the blots were stained with amido black (0.2% (w/v), 40% methanol, 10% glacial acetic acid) and destained. Blots

were then soaked in several changes of 100% methanol before being allowed to air dry.

2.5. Detection of biotinylated proteins

For detection of biotinylated polypeptides by the Neutravidin–Alexa Fluor430 conjugate, the PVDF blot was blocked in TBS-T containing 0.5% non-fat dry milk (NFDM) for 10 min prior to adding the conjugate at a concentration of 2.4 μg/ml. The blot was incubated for 45 min on an orbital shaker at 50 rpm at room temperature and washed 3 times for 5 min with TBS-T. For detection using the ECL-Plus system, the blot was blocked for 10 min in TBS-T containing 0.5% NFDM, then avidin-horseradish peroxidase conjugate (Avidin–HRP; 1 μg/ml) was added and incubated on an orbital shaker at 50 rpm for 45 min. The blot was washed 3 times for 5 min with TBS-T. ECL Plus substrate was then added to the blot following the recommendations of the manufacturer. For detection of biotinylated proteins using the ECF system, the blot was blocked for 10 min in TBS-T containing 0.5% NFDM, and then an avidin-alkaline phosphatase conjugate (Avidin–AP) was applied to the blot in TBS-T containing 0.5% NFDM at a dilution of 1:1000 for 45 min at room temperature. Following this incubation, the blot was washed in several changes of TBS-T. ECF substrate was then prepared and applied to the blot as directed by the manufacturer. Each blot was then analyzed on the Storm840.

2.6. Scanning and quantitation of biotinylated proteins

All blots were scanned for fluorescence by the Storm840 following the manufacturer's instructions. Once scanned, the blots were analyzed using ImageQuant software. Areas of interest were analyzed using a local average background correction. ImageQuant was then used to integrate the fluorescent intensity over each area.

3. Results

3.1. Detection and quantitation of biotinylated SDS-PAGE standards

Mixtures of biotinylated proteins of known molecular weight are available for use in western blotting systems that exploit the avidin–biotin interaction. These standards allow an analysis of the general applicability of these fluorescent detection systems to several distinct proteins, while still contained in a purified sample mixture. The standards mixture contained phosphorylase *b* (97,400 daltons), bovine serum albumin (66,200 daltons), ovalbumin (45,000 daltons), carbonic anhydrase (31,000 daltons), soybean trypsin inhibitor (21,500 daltons), and lysozyme (14,400 daltons). The degree of biotinylation and the concentration of each standard varies according to lot number, preventing absolute quantitation of the detection sensitivity. A two fold dilution series, beginning with the manufacturer's recommended preparation and ending with a 1:64 dilution of that sample, was resolved in triplicate on a 10% SDS–PAGE gel and transferred to polyvinyldifluoride (PVDF) as described in the Materials and Methods section. Each set of dilutions was then probed with the Neutravidin–Alexa 430 dye conjugate, avidin–horseradish peroxidase conjugate (ECL-Plus), or the avidin–alkaline phosphatase conjugate (ECF). The blots were then visualized with the appropriate fluorescent substrate, then scanned and quantified on the Storm840 instrument. The amount of fluorescence was then plotted against the magnitude of the dilution, along with the theoretical response if the system had exactly followed the serial dilution.

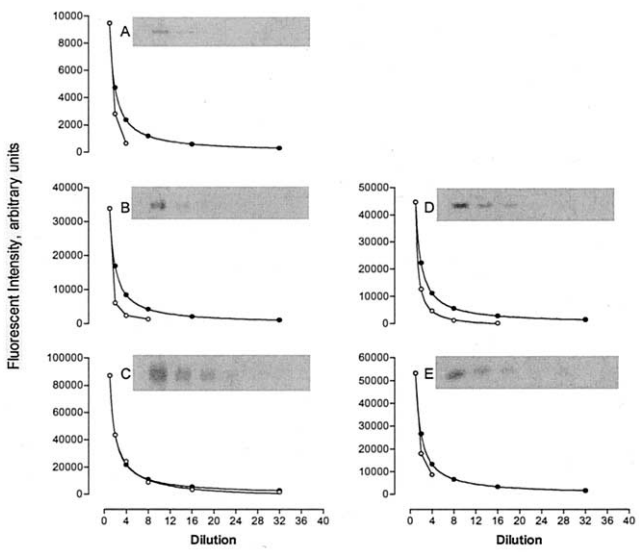

Fig. 1. Detection of biotinylated electrophoresis standards by Neutravidin–Alexa Fluor430. A serial dilution of biotinylated standards was resolved by SDS–PAGE and transferred to polyvinyldifluoride (PVDF) as described in the Materials and Methods section. The blots were then probed with Neutravidin–Alexa Fluor430 and scanned by the Storm840 optical scanner. Bands were then quantified using the ImageQuant for WindowsNT software. *Open circles*, reported Storm840 fluorescent intensity; *closed circles*, theoretical fluorescence response. A, phosphorylase b; B, bovine serum albumin; C, ovalbumin; D, carbonic anhydrase; E, soybean trypsin inhibitor.

For dilutions up to 32 fold, the response of the Neutravidin–Alexa Fluor430 conjugate was in good agreement (<10% discrepancy) with the theoretical response for all standards (Fig. 1). The lower limit of detection appeared to be different for many of the standards, but this is likely due to different initial concentration of each standard in the stock solution in order to achieve equal intensity on normal gel applications. In comparison, the response of the ECL-Plus system markedly deviated from the expected response for several of these standards, although the yield of fluorescence from this system was far greater than the Neutravidin –Alexa Fluor430 conjugate (Fig. 2). The response for carbonic anhydrase, soybean trypsin inhibitor, and ovalbumin was markedly different from expected, and in some cases

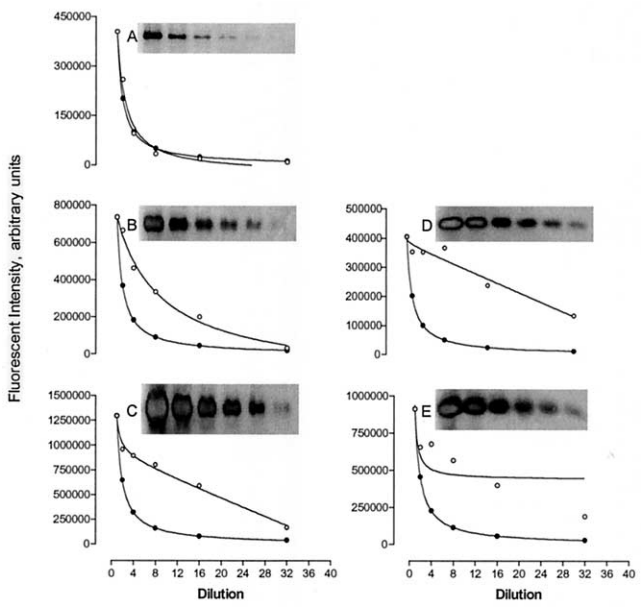

Fig. 2. Quantification of biotinylated electrophoresis standards by avidin–horseradish peroxidase conjugate and the ECL–Plus system. Biotinylated standards were separated as in Fig. 1 and detected using the ECL-Plus system. Fluorescently detected bands were quantified as in Fig. 1. *Open circles*, reported Storm840 fluorescent intensity; *closed circles*, theoretical response. A, phosphorylase b; B, bovine serum albumin; C, ovalbumin; D, carbonic anhydrase; E, soybean trypsin inhibitor.

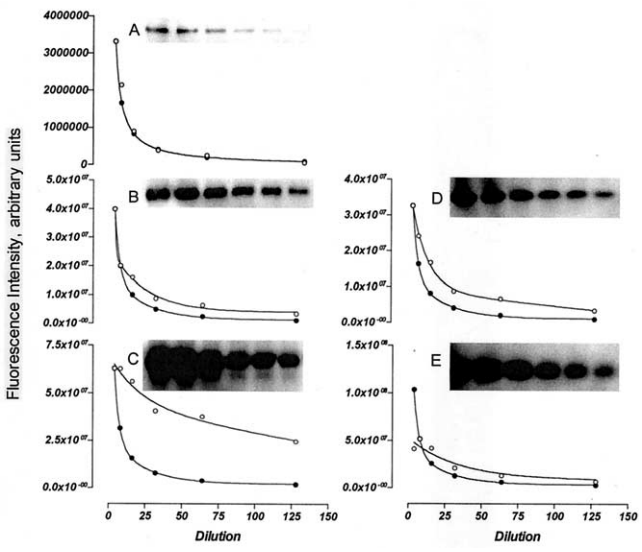

Fig. 3. Quantification of biotinylated electrophoresis standards by avidin–alkaline phosphatase conjugate and the ECF system. Biotinylated standards were separated as in Fig. 1 and detected using the ECF system. Fluorescently detected bands were quantified as in Fig. 1. *Open circles*, reported Storm840 fluorescent intensity; *closed circles*, theoretical response. A, phosphorylase b; B, bovine serum albumin; C, ovalbumin; D, carbonic anhydrase; E, soybean trypsin inhibitor.

the reported abundance was 1000% higher than expected (Fig. 2C, D, E). The detection of bovine serum albumin was closer to expected, while the detection of phosphorylase *b* was in very good agreement with the expected response. The ECF system exhibited a very strong signal, so that analysis of the biotinylated standards at dilutions less than 1:4 was prevented (Fig. 3). At dilutions from 1:4 to 1:125 however, the response of this system was in good agreement with the expected response with the exception of ovalbumin, which deviated markedly from expected response (Fig. 3C). The yield of fluorescence from this system was markedly higher than either the fluorescent avidin conjugate or the ECL-Plus system. Since it appeared that the poor performance of the ECL-Plus system might be related to sample loading, the response of the ECL-Plus and ECF system was evaluated at higher dilutions of the standards mixture. It should be noted that the Neutravidin–Alexa Fluor430 conjugate was not able to detect biotinylated standards at these higher dilutions, and therefore was not compared. When dilutions up to 16,000 were analyzed by these two systems, the observed response was in markedly better agreement with the expected serial dilution for all standards (data not shown).

3.2. Detection and quantitation of biotinylated bovine serum albumin

In order to obtain a more quantitative assessment of the sensitivity of the three techniques, biotinylated bovine serum albumin was used as a single standard of known concentration. Additionally, it is possible to determine the extent of biotinylation by measuring the molar ratio of biotin to target protein using a previously described method [14]. Using this technique, the biotinylation ratio of this protein was approximately 5 moles of biotin per mole of bovine serum albumin. Varying amounts of the biotinylated BSA were resolved in triplicate by SDS-PAGE and transferred to PVDF. Following transfer, the respective blots were probed with one of the three systems, visualized using the appropriate reagent system, and quantified on the Storm 840 instrument.

The Neutravidin–Alexa Fluor430 proved to be the least sensitive of the three methods, being able to detect to 0.245 ng biotinylated BSA, although the response of this system was in reasonably good agreement with expectations, even at relatively high loading (Fig. 4A). The ECL-Plus system exhibited much higher sensitivity, and was able to detect to 0.004 ng biotinylated BSA (Fig. 4B). The response of the ECL-Plus system was also in reasonably good agreement with the expected curve. The ECF system demonstrated the most sensitivity, as evidenced by the ability to detect 0.0009 ng biotinylated BSA. In addition, the response of the ECF system was remarkably close to the expected response (Fig. 4C).

3.3. Detection of liver biotinylated polypeptides

While the previous two analyses were performed on purified proteins or mixtures of purified proteins, applications of this technology would likely analyze biotinylated

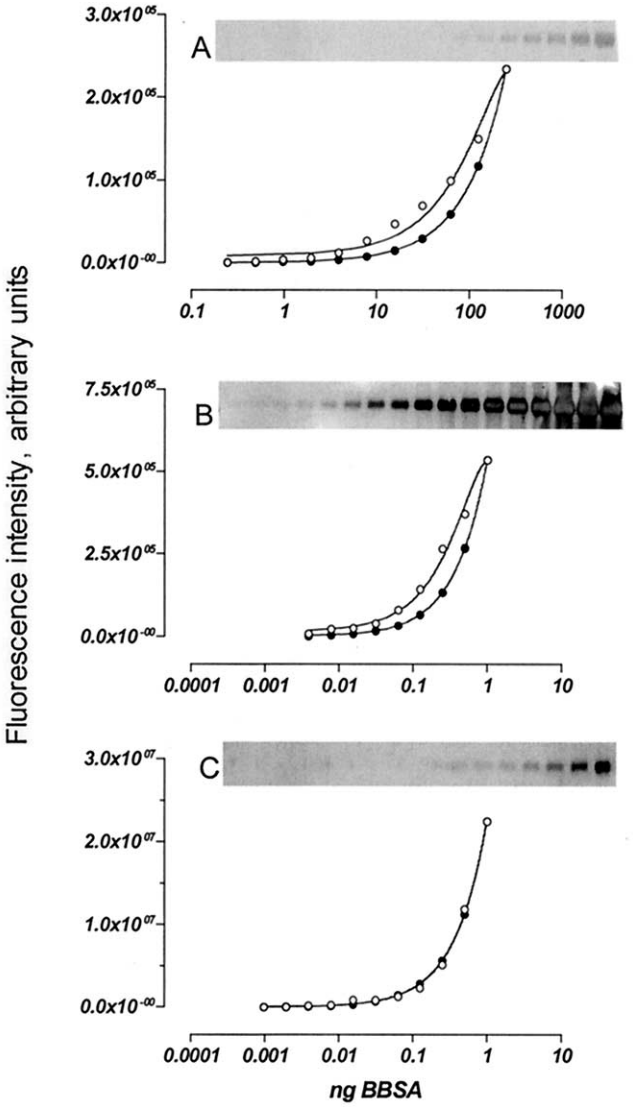

Fig. 4. Detection and quantification of biotinylated bovine serum albumin by three fluorescent methods. Biotinylated bovine serum albumin was generated and assessed as described in the Materials and Methods section. The biotinylated BSA was resolved by SDS–PAGE and transferred to PVDF. Each blot was then visualized using one of the three indicated detection system and was quantified using the ImageQuant for WindowsNT software and plotted against the mass resolved on the gel. A, avidin–AlexaFluor840 conjugate; B, avidin–horseradish peroxidase conjugate and the ECL-Plus system; C, avidin–alkaline phosphatase conjugate and the ECF system. *Open circles*, Storm840 fluorescent intensity; *closed circles*, theoretical response.

proteins in complex protein mixtures. In order to assess the detection and quantification of biotinylated proteins under these conditions, the linearity and sensitivity of the three detection systems was analyzed in rat liver extracts. Rat liver endogenously expresses the biotinylated enzymes acetyl CoA carboxylase isoforms 1 and 2, pyruvate carboxylase, methylcrotonyl CoA carboxylase, and propionyl CoA carboxylase. Although the exact abundance of these proteins is not known in relation to the total amount of protein resolved by the gel, the molar ratio of protein biotinylation is known to be 1:1, since only one biotinylation site exists in each of these enzymes [17]. As above, various amounts of rat liver homogenate extracts (100–1.56 μg by serial dilution for Neutravidin–Alexa Fluor430 and the ECL-Plus system, 25–0.091 μg by serial dilution for the ECF system) were resolved and transferred to PVDF prior to being probed by one of the detection systems. Blots were then scanned and quantified using the Storm 840 instrument.

The avidin-Alexa Fluor430 conjugate was able to detect pyruvate carboxylase, propionyl CoA carboxylase, and methylcrotonyl CoA carboxylase, but failed to detect either acetyl CoA carboxylase isoform 1 or 2 under these conditions. For the carboxylases that were detected, however, the observed response was linear for pyruvate carboxylase and fit a second order polynomial curve for the methylcrotonyl and propionyl CoA carboxylase bands (Fig. 5A). There were, however, significant background bands that were not competed by incubation with free biotin (data not shown). As expected, the ECL-Plus system exhibited a much higher yield of fluorescence, but sensitivity was not likewise markedly increased for any of the carboxylases (Fig. 5B). Again, the ECF system exhibited by far the highest yield of fluorescence and sensitivity compared to the Neutravidin–Alexa Fluor430 and the ECL-Plus system (Fig. 5C).

4. Discussion

The aim of the present study was to validate fluorescent detection systems for use in the quantification of biotinylated polypeptides, and to determine under what conditions such quantification would be reliable. The results of these experiments should greatly facilitate optimization of experimental conditions for such detections in a variety of applications. It should be noted that the visual representation of the blots is irrelevant in these analyses since the contrast of these pictures can be arbitrarily altered without changing the underlying data that the Storm840 system reports. In these studies, we have assessed the reliability of each system by analyzing how the detection of serial dilutions responded in comparison to how each system should have theoretically behaved; i.e., given a specific amount of fluorescence from a biotinylated protein band, loading half as much protein should result in half the detected fluorescence. In this way, the ability of each system to discriminate between samples with varying abundances of biotinylated polypeptides can be directly assessed. For example, Detecting biotinylated polypeptides using the Neutravidin–Alexa Fluor430 conjugate has the potential advantage of not relying upon the enzymatic activity of either alkaline phosphatase or horseradish peroxidase and the associated kinetic characteristics to confound the analysis. However, this same characteristic presents a potential drawback since there is no amplification possible to enhance the signal. In agreement with our expectations, the response of the fluorescent avidin conjugate was very similar to the theoretical response of the serial

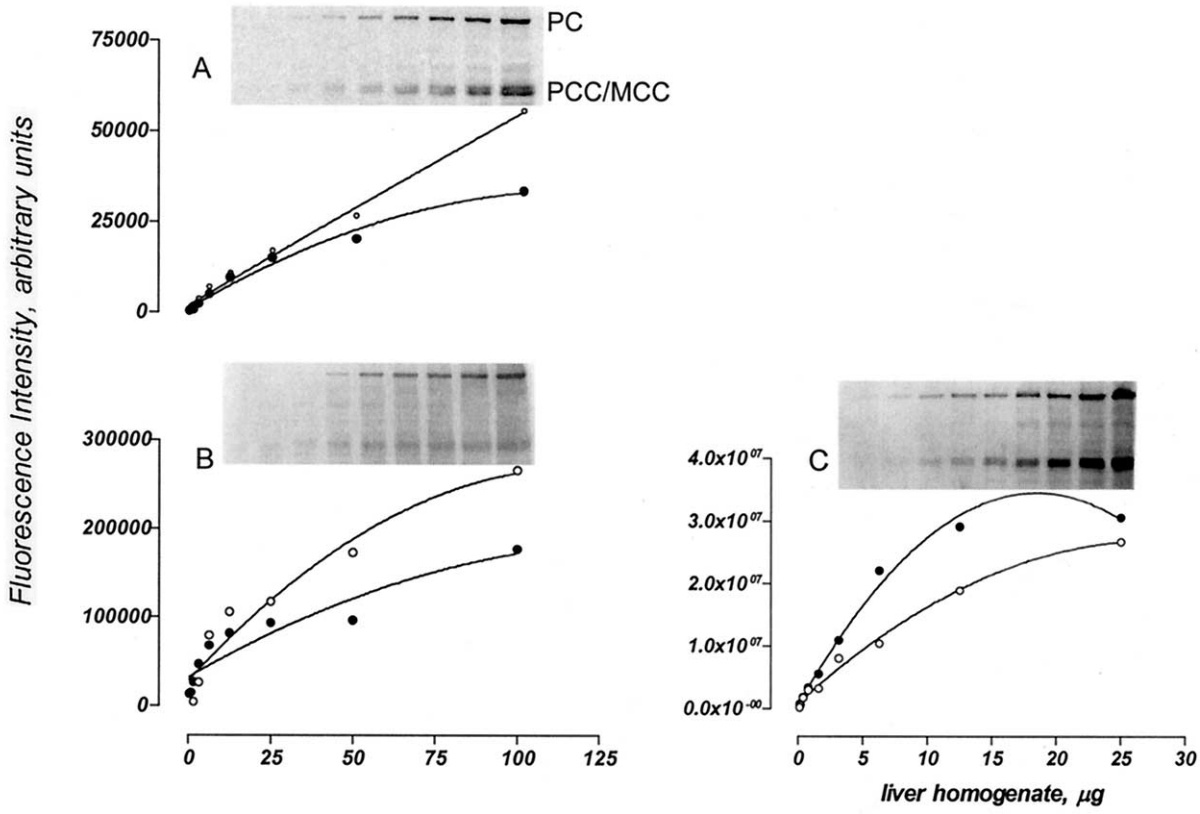

Fig. 5. Detection and quantification of biotinylated polypeptides in rat liver homogenate. Various amounts of rat liver homogenate were resolved by SDS–transferred to PVDF. Blots were then probed with one of the three indicated systems and visualized on the Storm840 instrument and quantified using the ImageQuant densitometric software as described in the Materials and Methods section. The fluorescent intensity is plotted against the amount of rat liver homogenate resolved by gel electrophoresis. A, avidin–Alexa Fluor430 conjugate; B, avidin–horseradish peroxidase and ECL; C, avidin–alkaline phosphatase and ECF. *Open circles*, pyruvate carboxylase; *closed circles*, propionyl CoA carboxylase and methylcrotonyl CoA carboxylase combined intensity.

dilution, since the relationship between fluorescence emitted and amount of Neutravidin–Alexa Fluor430 conjugate bound should be stoichiometric. However, we observed that the avidin system did not exhibit the sensitivity of the two enzyme linked detection systems. Under medium to high expression levels or protein loading levels, this system should prove reliable.

The emission spectrum of the fluorescent product of the ECL-Plus system has a maximum of 460 nm, significantly less than the optimal detection spectrum of the Storm840. We therefore expected that the detection of biotinylated proteins would be significantly diminished. To our surprise, although the yield of detected fluorescence was not as high as in the ECF system, the detection system proved quite sensitive. One limitation of this system is that at high loading densities, bands detected by the ECL-Plus system had an "ghosted" appearance, indicating a loss of signal in the interior of large dense bands. The response of this system, however, improved markedly at low protein loadings.

As we expected, the ECF detection system displayed the highest sensitivity of the three systems, in agreement with its relatively high fluorescent yield and its coincidence with the detection parameters of the Storm840. Like the ECL-PLUS system, the ECF system had some difficulty in assessing biotinylated proteins at relatively high protein loading, though this difficulty was less pronounced. We have noticed a slight tendency for the fluorescent product in the ECF system to diffuse if care is not taken not to disturb the blot while on the Storm840 instrument; and we believe that the soluble nature of this product prevents the "inversion" effect observed in the ECL-Plus system. Nonetheless, the ECF system demonstrated the highest sensitivity while maintaining reasonable reliability at high protein loading and excellent reliability at low protein loading.

The apparent error in quantification observed in some of the experiments appeared to be associated with the amount of protein on the blot in relation to the sensitivity of the visualization system, rather than a general limitation of using the Storm 840 instrument in general. Supporting this conclusion is the extremely wide dynamic range of the Storm 840 that is clearly superior to either colorimetric or film based approaches. Additionally, systems that exhibited poor performance could be vastly improved by changing the protein loading. Therefore, it is likely that the behavior of the particular fluorescent product and the manner in which it accumulates on the blot at the site of reactivity is the cause of the error. As one example, it is possible that the "ghosting" phenomenon observed in the middle of some bands

(where there is reduced fluorescence despite a large amount of target protein) is that accumulation of product at the site is so large that the "pile" of product actually inhibits either its excitation by the instrument or its resulting emission. Aside from these conditions, however, these systems can provide very accurate quantitation given careful attention to the loading parameters.

The Storm840 optical scanner is a common instrument in many modern molecular biology laboratories due to its ability to measure medium and high energy radioactivity through storage phosphor imaging technology. Our studies on the suitability of this instrument in the fluorescent analysis of biotinylated polypeptides should significantly extend its capabilities without extensive modification.

References

[1] Santiago MP, Potter LT. Brain Res 2001;894:12–20.
[2] Guan YH, de Graaf T, Lath DL, Humphreys SM, Marlow I, Brook AH. Arch Oral Biol 2001;46:129–38.
[3] Shalamanova L, Kubler B, Scharf JG, Braulke T. Growth Horm IGF Res 2000;10:294.
[4] Sequeira H, Poulain P, Ba-M'Hamed S, Viltart O. Brain Res Brain Res Protoc 2000;5:49–56.
[5] Ryder JW, Yang J, Galuska D, Rincon J, Bjornholm M, Krook A, Lund S, Pedersen O, Wallberg-Henriksson H, Zierath JR, Holman GD. Diabetes 2000;49:647–54.
[6] Magnusson S, Hou M, Hallberg EC, Breimer ME, Wadenvik H. Thromb Res 1998;89:53–8.
[7] Hofstetter H, Morpurgo M, Hofstetter O, Bayer EA, Wilchek M. Anal Biochem 2000;284:354–66.
[8] Vaitaitis GM, Sanderson RJ, Kimble EJ, Elkins ND, Flores SC. Biotechniques 1999;26:854–8.
[9] Praul CA, Brubaker KD, Leach RM, Gay CV. Biochem Biophys Res Commun 1998;247:312–4.
[10] Santos PR, Chaves ME. Braz J Med Biol Res 1997;30:837–42.
[11] Kin SH, Suh YH. Comp Biochem Physiol B Biochem Mol Biol 1996;115:57–61.
[12] Hoeltke HJ, Ettl I, Strobel E, Leying H, Zimmermann M, Zimmermann R. Biotechniques 1994;18:900–4, 906–7.
[13] Dunn MJ. Methods Mol Biol 1994;32:227–32.
[14] Green NM. Biochemical Journal 1965;94:23c–24c.
[15] Markwell MA, Haas SM, Bieber LL, Tolbert NE. Anal Biochem 1978;87:206–10.
[16] McMahon RJ, Cousins RJ. Proc Natl Acad Sci USA 1998;95:4841–6.
[17] Chapman-Smith A, Cronan JE Jr. Trends Biochem Sci 1999;24:359–63.