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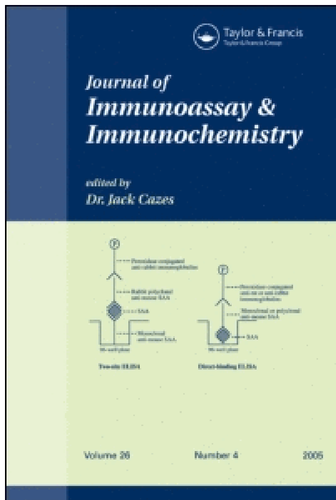

Journal of Immunoassay and Immunochemistry
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To cite this Article Kamiya, Toshio(1997) 'A Procedure for the Detection of Free Thiol-Containing Proteins on a Polyvinylidene Difluoride Membrane', Journal of Immunoassay and Immunochemistry, 18: 1, 111 — 123
To link to this Article: DOI: 10.1080/01971529708005807
URL: http://dx.doi.org/10.1080/01971529708005807

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JOURNAL OF IMMUNOASSAY, 18(1), 111-123 (1997)

A PROCEDURE FOR THE DETECTION OF FREE THIOL-CONTAINING PROTEINS ON A POLYVINYLIDENE DIFLUORIDE MEMBRANE

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ABSTRACT

Bovine serum albumin (BSA), which has a free thiol, was blotted onto a polyvinylidene difluoride membrane. The membrane was reacted with a sulfhydryl-reactive (maleimide-containing) biotin derivative, 1-biotinamido-4-[4'-(maleimidomethyl)cyclohexanecarboxamido] butane (Biotin-BMCC), and then probed. BSA on membranes was detected semi-quantitatively at 50 ng of protein (0.76 pmol of free thiol) and among range of higher extent. BSA on membranes was less efficiently biotinylated compared with biotinylation in solution. Regardless, these results suggested that sulfhydryl-containing proteins were specifically and semi-quantitatively identified by membrane biotinylation with Biotin-BMCC.
(KEY WORDS: BSA; membrane biotinylation; PVDF; thiol)

INTRODUCTION

There are a number of reports on the chemical cross-linking of proteins for their detection (labeling) and drug conjugation (1-10). Biotinylation of proteins, including antibody and ligand, is widely used for preparation of molecular probes (11, 12). Several reagents for biotinylation are available. One of them, 1-biotinamido-4-[4'-(maleimidomethyl)cyclohexanecarboxamido] butane

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(Biotin-BMCC[1]), is a sulfhydryl-reactive (maleimide-containing) biotin derivative to biotinylate a sulfhydryl group of cysteine residue.

Quantitative measurement of thiol groups has been made possible by spectrophotometry, using 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and organic mercuric halide as *p*-chloromercuribenzoate (PCMB) (13). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by protein blotting, on the other hand, is a powerful tool for identification of proteins. It has been reported that proteins blotted to solid-phase membranes can be detected directly by membrane biotinylation (14). However, it remains to be clarified whether sulfhydryl-containing proteins on a solid phase are identified directly and specifically with a thiol-specific cross-linking reagent. Therefore, I have tried to determine whether cysteine residue of bovine serum albumin (BSA) immobilized on a polyvinylidene difluoride (PVDF) membrane is biotinylated with Biotin-BMCC and identified.

MATERIALS AND METHODS

Chemicals and Reagents

BSA was purchased from Sigma (St. Louis, MO, U.S.A.). Biotin-BMCC and N,N'-diacetyl-1,6-hexanediamine were purchased from

[1]Abbreviations used: ANOVA, analysis of variance; Biotin-BMCC, 1-biotinamido-4-[4'-(maleimidomethyl)cyclohexanecarboxamido]butane; BSA, bovine serum albumin; D-PBS, Dulbecco's phosphate-buffered saline; DMSO, dimethylsulfoxide; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); PCMB, *p*-chloromercuribenzoate; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Pierce (Rockford, IL, U.S.A.) and Lancaster (Morecambe, Lancashire, England), respectively. Dimethylsulfoxide (DMSO) was purchased from Sigma and Nacalai Tesque (Kyoto, Japan). PVDF membranes and avidin/biotinylated peroxidase complex were purchased from Millipore (Bedford, MA, U.S.A.) and Vector (Burlingame, CA, U.S.A.), respectively. SDS-PAGE molecular weight standards (low range) were purchased from Bio-Rad (Hercules, CA, U.S.A.), including rabbit muscle phosphorylase b (molecular weight, 97.4 kDa), BSA (66.2), hen egg white ovalbumin (45.0), bovine carbonic anhydrase (31.0), soybean trypsin inhibitor (21.5), and hen egg white lysozyme (14.4).

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Protein Blotting

The samples were incubated for 5 min at 90°C in Laemmli's sample buffer with or without 5% 2-mercaptoethanol and electrophoresed on a 10-20% polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) (15). The proteins were transferred to PVDF membranes at 30 V for 12 to 15 h at 4°C (16). After washing three times for 30 min with D-PBS containing 0.1% Tween 20, the membranes were incubated for at least 30 min at room temperature with avidin/biotinylated peroxidase complex. The membranes were then washed three times for 30 min with D-PBS containing 0.1% Tween 20, and the proteins recognized were visualized by ECL Western blotting detection reagents (Amersham, Little Chalfont, Buckinghamshire, England).

Biotinylation

To biotinylate proteins in solution, aliquots of the proteins

(BSA 50μg/ml) were 10-fold diluted and incubated with 93-94 molar excess of Biotin-BMCC (7 μM in D-PBS) for 2 h at room temperature. The biotinylated proteins were then processed as above.

Biotinylation of proteins on a membrane was done as follows: The samples were subjected to SDS-PAGE and blotting as above. The blotted membrane was incubated with Biotin-BMCC (10 μM in D-PBS) for 30 min at room temperature and washed for 30 min with D-PBS containing 10% dimethylsulfoxide and then with D-PBS containing 0.1% Tween 20, and then processed as above. The membranes were treated with N,N'-diacetyl-1,6-hexanediamine (0.5 M in D-PBS) before and after biotinylation to reduce high background signals probably due to nonspecific adsorption of Biotin-BMCC to membranes.

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Densitometric Analysis

A series of exposures to films were made, and the films on which the lowest concentration of BSA was just detectable were used for densitometric analysis. The film was scanned using a densitometer (Hiranuma DM-301K; Tokyo, Japan). A graph of peak area (optical density unit) was plotted against the amount of free thiol of BSA (pmol). A dose-response curve was obtained by the least squares method. Significant linearity between 0.76-26 pmol was confirmed by analysis of variance (ANOVA)/regression analysis.

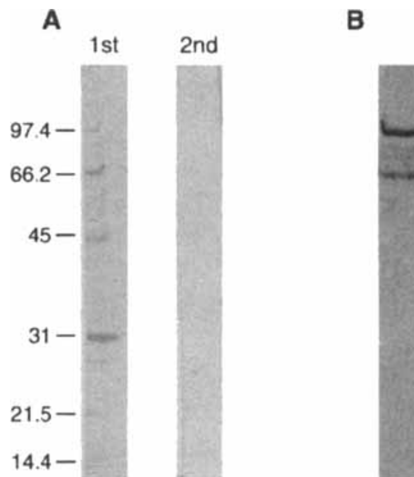

FIGURE 1: Blotting efficiency. **A,** After placing two membranes to the gel following electrophoresis and the current on the electroblotting device was applied, both the membrane (1st) placed in contact with the gel and the membrane (2nd) placed in contact with the 1st membrane were stained with Coomassie Brilliant Blue R-250 and destained. **B,** The gel was stained with silver.

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RESULTS AND DISCUSSION

Blotting Efficiency

Transfer conditions for BSA were determined using molecular weight standards on the length of time the current was applied. When placing two pieces of membrane, one of which was in contact with the gel following electrophoresis, and the current on the electroblotting device was applied for 12 to 15 h (at 30 V at 4°C), transferred proteins were detected more extensively on the first membrane than the second. The silver-stained gel, on the

other hand, showed only trace band(s) of BSA (and phosphorylase b). These results indicated that this condition was preferable among tested (Fig. 1).

Biotinylation on a Membrane

Biotinylation of proteins on a membrane was done. Fifty and 250 ng of BSA were subjected to SDS-PAGE and blotting as above. The blotted membrane was incubated with 10 μM to 1 mM of Biotin-BMCC (in D-PBS) for 30 min or 2 h at room temperature, and the biotinylated proteins were probed with avidin/biotinylated peroxidase complex (Table 1). BSA was detected at 250 ng and faintly at 50 ng (Fig. 2A) even under the condition of biotinylation with 10 μM of Biotin-BMCC for 30 min at room temperature. This condition showed lesser extent of background signals than others (Table 1). These high background signals seemed to be due to nonspecific adsorption of Biotin-BMCC to membranes. DMSO used as a solvent for Biotin-BMCC was added to washing buffer. Membrane biotinylation experiments, without blots, showed that both membranes treated with 10 or 100 μM of Biotin-BMCC for 30 min at room temperature, followed by washing with D-PBS in the presence or absence of 10% DMSO, gave lesser extent of background signals in contrast to those followed by washing with DMSO or D-PBS containing 50% DMSO (Table 1). The above blots were then washed for additional 30 min with or without D-PBS containing 10% DMSO, followed by washing with D-PBS containing 0.1% Tween 20. These treatments reduced background

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TABLE 1

Membrane Biotinylation under Various Conditions

	Biotin-BMCC (μM, hr)	Washing Buffer[1]	Background Signal[2]	Sensitivity (ng)[3]
Exp1	10, 2[4]	A	++++	>250
	10, 0.5[5]	A	+++	250
	100, 0.5[6]	A	++++	>250
	1000, 0.5[7]	A	+++++	>250
	(4)	A	++	250
	(5)	B, A	+	250
	(6)	B, A	++	250
	(7)	A	+++	50
Exp2	10, 0.5	B, A	+	50
Exp3[8]	10, 0.5	A	+	50
Exp4	F[9];10, 0.5	F, B, A	+	>250
	F;10F[10],0.5	F, B, A	+	>250
Exp5	F;10F,0.5	F, B, A	+	250
Exp6	10, 0.5	A	+	ND[11]
	10, 0.5	B, A	+	ND
	10, 0.5	C, A	+/++	ND
	10, 0.5	D, A	+/++	ND
	100, 0.5	A	+/++	ND
	100, 0.5	B, A	+	ND
	100, 0.5	C, A	++	ND
	100, 0.5	D, A	++	ND
Exp7	10, 0.5	(E;B);A[12]	+	ND
	E;10E,0.5	(E; B); A	+	ND
	100, 0.5	(E; B); A	++	ND
	E;100E,0.5	(E; B); A	++	ND
Exp8	14, 0.5	(F; B); A	++	ND
	F;14F,0.5	(F; B); A	++	ND
	140, 0.5	(F; B); A	++	ND
	F;140F,0.5	(F; B); A	++	ND

1) A, D-PBS+Tween; B, D-PBS+10% DMSO; C, D-PBS+50% DMSO, D; DMSO; E, 10 mM analog; F, 0.5 M analog.
2) + to +++++ denote lower to higher backgrounds.
3) The lowest concentration of BSA just detectable.
4,5,6,7) The blots were processed, respectively.
8) Photos were shown in Fig. 2A.
9) Pretreated with F.
10) Biotinylated with 10 μM Biotin-BMCC in F.
11) Not determined: Membranes, to which BSA was not immobilized, were used.
12) Treated with E and/or B, and A.

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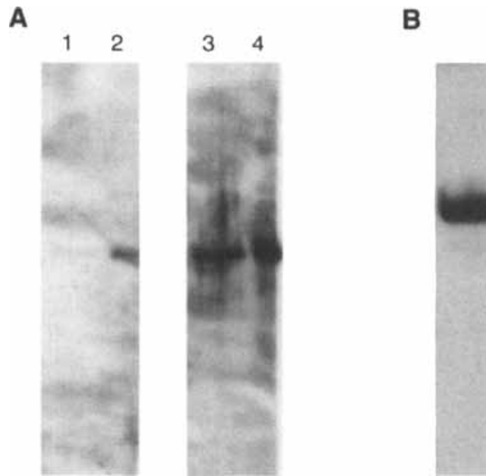

FIGURE 2: Biotinylation of proteins on a membrane. **A,** Fifty (lanes 1, 3) and 250 ng (lanes 2, 4) of BSA were subjected to SDS-PAGE and blotting, and the blotted membrane was incubated with 10 µM of Biotin-BMCC (in D-PBS) for 30 min at room temperature and the proteins were probed with avidin/biotinylated peroxidase complex. BSA run under reducing (lanes 3, 4) and non-reducing conditions (lanes 1, 2) are shown. **B,** BSA (50 ng) in solution (10 µl) biotinylated with 93-94 molar excess of Biotin-BMCC (7 µM in D-PBS) for 2 h at room temperature.

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signals without any loss of sensitivity (Table 1). These results indicated that the preferable conditions of biotinylation of proteins on membranes were with 10 or 100 µM of Biotin-BMCC for 30 min at room temperature, followed by washing occasionally with D-PBS containing 10% DMSO and then by washing with D-PBS containing 0.1% Tween 20. However, 50 ng of BSA in solution (10 µl) biotinylated with 93-94 molar excess of Biotin-BMCC (7 µM in D-PBS) for 2 h at room temperature as described under "Materials and Methods" showed a higher signal-to-noise ratio (Fig. 2B).

Reduction of Nonspecific Adsorption of Biotinylating Reagents to a Membrane

Hydrophobic interaction between Biotin-BMCC, which has a structure consisting of N-(butane-carboxamido)-N'-(cyclohexane-carboxamido)-1,4-butanediamine, and PVDF membrane components might lead to nonspecific adsorption of Biotin-BMCC to membranes. An analog of this structure, N,N'-diacetyl-1,6-hexanediamine was then used to reduce background signals. The membranes were treated with 10 or 500 mM of this analog before and after biotinylation, indicating that these treatments reduced adsorption of Biotin-BMCC to the membranes (Table 1). However, detection of 50 ng of biotinylated BSA failed, probably because the adsorbed analog inhibited accessibility of Biotin-BMCC to free thiol of BSA on membranes. Data obtained so far were summarized in Table 1.

Semi-Quantitative Detection

Fifty and 250 ng of BSA were subjected to blotting following SDS-PAGE under reducing (26 and 132 pmol of free thiol, respectively) or nonreducing (0.76 and 3.8 pmol of free thiol, respectively) condition. The blotted membranes were incubated with 10 μM Biotin-BMCC (in D-PBS) for 30 min at room temperature, and the biotinylated proteins were probed with avidin/biotinylated peroxidase complex. A series of exposures to films were made. The films on which the lowest concentration of BSA was just detectable were used for densitometric analysis. The

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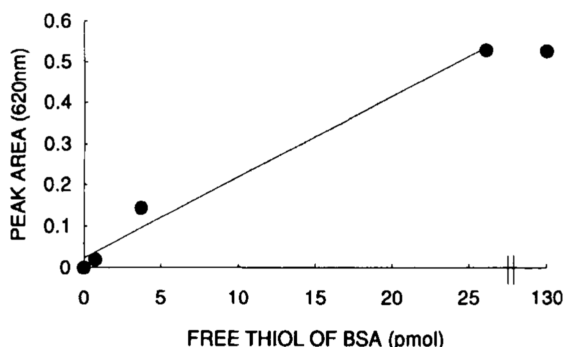

FIGURE 3: Semi-quantitative analysis of free thiol by membrane biotinylation. The film (Fig. 2A) was scanned using a densitometer and a graph of peak area (optical density unit) was plotted against the amount of free thiol of BSA (pmol). A dose-response curve was obtained by the least squares method. Significant linearity between 0.76-26 pmol was confirmed by analysis of variance (ANOVA)/regression analysis.

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film (Fig. 2A) was scanned using a densitometer. A graph of peak area (optical density unit) was plotted against the amount of free thiol of BSA (pmol) (Fig. 3). A dose-response curve was obtained by the least squares method. Significant linearity between 0.76-26 pmol was confirmed by ANOVA/regression analysis, indicating quantification of BSA on solid phase membranes with the sulfhydryl-reactive reagent. These results suggested that sulfhydryl-containing proteins were specifically and semi-quantitatively identified by biotin/avidin system and membrane biotinylation with a thiol-specific cross-linking reagent following SDS-PAGE and protein blotting.

This technique could make it possible to determine protease susceptibility of only hundreds ng of a protein having respectively protease recognition sequences after or before free

thiol at N- or C-terminal ends, without amino acid sequencing (proteins of μg order are required). In this context, Koritsas, V.M. and Atkinson, H.J. (17) have recently reported a solid-phase proteinase assay based on using as substrate biotinylated gelatin adsorbed onto microtiter plates. Furthermore, membrane biotinylation following protease treatment could allow identification of cysteine residues of proteins not responsible for disulfide bonding. Biotinylation in solution would alter the molecular properties including molecular weight. On the other hand, Sulter, M.W. *et al.* (18) developed a solid-phase protein assay based on biotinylation of immobilized protein on the microtiter plate and its subsequent quantitation by biotin/avidin system for determination of protein concentration. Biotinylation of immobilized protein on the microtiter plate with a thiol-specific cross-linking reagent could permit a quantitative measurement for thiol.

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ACKNOWLEDGEMENTS

I thank Drs. T. Masuko (Tohoku University) and Y. Hashimoto (Tohoku University) for discussions, valuable suggestions, and critical review of the manuscript; Dr. S. Mochizuki (this laboratory) for technical advice of densitometric analysis; Drs. K. Yokoyama (The Green Cross Corporation), Y. Kagitani, and K. Yamanouchi (this laboratory) for valuable counsel; Dr. K. Okabayashi (this laboratory) for encouragement; Dr. M. Tsujikawa (this laboratory) for discussions, suggestions, and critical review of the manuscript.

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