Biotin–Protein Ratios and Stability of Biotinylated Immunoglobulins as Standards for the Quantitation of Biotin-Binding Immunoglobulins

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Biotin-binding IgG in human sera was quantitated using F(ab')$_2$anti-human IgG-coated multiwell microplates (Muratsugu, M. *et al.* 2003, *Biol. Pharm. Bull.*, 26, 1605—1608). The biotin–protein ratio of biotinylated IgG, which was used as standard in the assay, was very important to quantitate the level of biotin-binding IgG. We investigated a synthesis method of biotinylated human immunoglobulins, how to determine the biotin–protein ratio of the biotinylated proteins, and their stability to prepare standards for measuring biotin-binding IgG, IgA, and IgM.

Key words biotinylated protein; biotin–protein ratio; stability; competitive binding assay; bioassay; biotin-binding immunoglobulin

Immunoglobulins to which biotin was covalently linked were found in human sera for the first time in 1993.[1)] The prevalence of biotin-binding immunoglobulin was higher in patients with atopic dermatitis, other dermatitis, allergic disorders, and autoimmune disease.[1—3)] Recently, biotin-binding IgG was detected in a multiwell microplate format for the first time.[4)] We developed a quantitative assay for biotin-binding IgG without any purification of samples using F(ab')$_2$anti-human IgG-coated multiwell microplates.[5)] We showed that the ratio of biotin molecules to protein molecules (biotin–protein ratio) in biotinylated IgG in human sera affected the detection limit in this assay.[5)] The slope of dose-response curve of the assay decreased as the biotin–protein ratio of standards decreased and the observed values of biotinylated IgG in human sera depended on the biotin–protein ratio of standards. Thus it was very important to measure the biotin–protein ratio of biotinylatd immunoglobulins which were used as standards in the assay.

In this study, we investigated a synthesis method for biotinylated human immunoglobulins, how to determine the biotin–protein ratio of the biotinylated proteins, and their stability.

MATERIALS AND METHODS

Materials The following materials were obtained from the sources as indicated: flat-bottomed multiwell microplates (Immulon I) from Dynatech Laboratories, Inc., U.S.A.; *N*-hydroxysuccinimidobiotin (NHS-biotin) and BCA Protein Assay Reagent kit from Pierce Chemical Co., Rockford, U.S.A.; biotin, horseradish peroxidase (HRP)-labeled streptavidin, dimethyl sulfoxide (DMSO), human IgG, human IgA, human IgM, and BSA from Sigma Chemical Co., St. Louis, U.S.A.; ELISA Color Reagent kit (Type O) from Sumitomo Bakelite Co., Ltd., Tokyo, Japan; Biotin Assay Medium® from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; Microcon YM-3 from Millipore Co., Beldford, U.S.A. All other chemicals were of reagent grade or better. The water used was 17-Mohm grade.

Biotinylation of IgA, IgM, IgG, and BSA Biotinylation of BSA was also examined, since biotinylated BSA was used for the quantitation of biotin in this study. Twenty-five microliters of NHS-biotin DMSO solution at various concentrations was added to 1 ml of 2 mg ml^{-1} IgA, IgM, IgG, or BSA in 50 mmol l^{-1} sodium bicarbonate/HCl buffer (pH 8.6). The mixture was gently stirred at room temperature for a fixed time. The reaction solution was dialyzed against 0.15 mol l^{-1} NaCl and finally against Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS). The amounts of biotinylated IgA, IgM, IgG, and BSA were expressed as the amounts of IgA, IgM, IgG, and BSA, respectively, which were determined using a BCA Protein Assay kit with human IgG and BSA as standards for biotinylated IgA, IgM, and IgG and for BSA, respectively.

Quantitation of Biotin Using Biotinylated BSA-Coated Multiwell Microplate The principle of biotin quantitation was the same as has been previously reported.[6—9)] We used the following procedure with a small amount of biotinylated BSA, which was different from the procedure reported previously.[6,7)] Multiwell microplates (96 wells) were coated with biotinylated BSA by adding 50 μl of 0.1 μg ml^{-1} biotinylated BSA in PBS and 0.02% NaN_3 (PBS-N) to each well, then incubating overnight at 6 °C. After the wells were washed five times with PBS-N, 300 μl of 1% BSA in PBS-N was added to each well. The plates were incubated at 37 °C for 2 h and washed three times with PBS-N. Fifty microliters of test solutions or standards were added to each well and 50 μl of HRP-streptavidin was then added. The plates were incubated at 37 °C for 2 h and washed five times with PBS-N and twice with PBS containing 0.05% merthiolate (PBS-M). One hundred microliters of substrate solution in an ELISA Color Reagent kit was added to each well. After the plate was incubated at 25 °C for 20 min, the color reaction was terminated by addition of a reaction stop solution in the kit to each well. Absorbance at 490 nm was then measured in a microplate reader (Model 450, Bio-Rad Labs., Richmond, U.S.A.) and the data were transferred to a computer using a home-made program for data analysis.[10)] The competitive binding assay used was referred to as CBA in the study.

Quantitation of Biotin by Agar Plate Bioassay Fukui *et al.*[11)] previously developed an agar plate biotin bioassay (BA) method using *Lactobacillus plantarum* ATCC8014, a biotin-dependent microorganism. Briefly, a small aliquot of samples was added to each well on the agar plate containing

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Biotin Assay Medium® and the agar plate was incubated at 37 °C for about 16 h. The diameter of the growth zone was measured and compared with the standard curve.

Estimation of Biotin–Protein Ratio of Biotinylated IgA, IgM, and IgG Biotin–protein ratios of biotinylated IgA, IgM, and IgG were estimated by multiwell microplate method as previously reported.[5] Briefly, Multiwell microplates were coated with F(ab')$_2$anti-IgA. Biotinylated IgA was bound to immobilized F(ab')$_2$anti-IgA. The avidin-biotinylated peroxidase complex reacted to the biotinylated IgA and an enzyme reaction occurred after addition of substrate. The color intensity depended on the quantity of biotinylated IgA, but increased in proportion to the biotin–protein ratio when the amount of biotinylated IgA was constant (data not shown). Biotinylated IgM and IgG could be quatitated using the same procedure. The assay was stated as an F(ab')$_2$anti-Ig assay in this study.

Determination of Biotin–Potein Ratio of Biotinylated Proteins The biotin–protein ratio of each biotinylated protein was determined as follows: Biotinylated IgA, IgM, IgG, and BSA were freeze-dried and the freeze-dried proteins were hydrolyzed in 6 mol l^{-1} HCl in the gas phase at 110 °C for 24 h. The acid-hydrolyzed proteins were redissolved in 0.02 mol l^{-1} HCl and the solutions were diluted with PBS-M for the quantitation of biotin. The amount of biotin molecules released from the biotinylated proteins was measured by CBA and BA, and those of proteins were measured by BCA Protein Assay kit as stated above. The molecular weights of IgA, IgM, IgG, and biotin were 160000, 900000, 150000, and 244, respectively.[12]

Recovery of biotin in the above procedure was investigated. Biotin in PBS was freeze-dried and treated in 6 mol l^{-1} HCl in the gas phase at 110 °C for 24 h. The mixture was redissolved in 0.02 mol l^{-1} HCl and the solutions were diluted with PBS-M for the biotin quantitation. Biotin molecules were measured by CBA.

Stability of Biotinylated Proteins Two hundred microliters of biotinylated IgG or BSA solution was centrifuged at 12000×$\boldsymbol{g}$ using Microcon YM-3 (the nominal molecular weight limit=3000) at 4 °C for 1 h (TMA-2 rotor, High speed micro refrigerated centrifuge MR-150, Tomy, Tokyo, Japan). The levels of biotin in a dilution series of the filtrate were analyzed by CBA (State 1). Absorbances obtained were almost the same. Biotinylated IgG or BSA was stored under different conditions. After the samples were stored for a fixed time, they were centrifuged under the same centrifugation conditions and levels of biotin in a dilution series of the filtrate were analyzed by CBA (State 2). Absorbance at State 1 was taken as an arbitrary unit of 1. Absorbance at a dilution (State 2) was compared with that at the same dilution (State 1). The results were expressed as arbitrary units (absorbance (State 2)/absorbance (State 1)).

RESULTS AND DISCUSSION

Correlation between Biotin Levels Measured by CBA and BA Concentrations of biotin in the samples were measured by CBA ([Biotin]$_{CBA}$) and BA ([Biotin]$_{BA}$) and compared (Fig. 1). The correlation coefficient was 0.997, indicating a good correlation between [Biotin]$_{CBA}$ and [Biotin]$_{BA}$. CBA used in this study showed that the biotin level was almost the same as that measured by BA when utilizing *L. plantarum*, the most widely used microorganism in biotin bioassays.

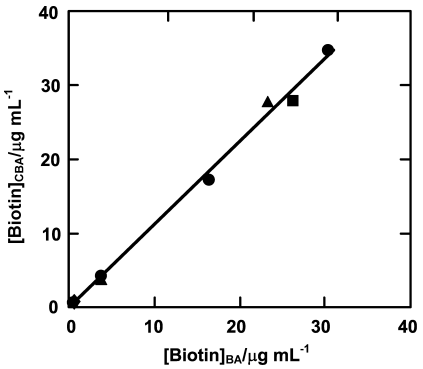

Fig. 1. Correlation between Biotin Levels Quantitated with CBA and BA

The biotin concentrations were measured with CBA and BA, after B-IgA (●), B-IgM (▲), B-IgG (■), and B-BSA (◆) were acid-hydrolyzed. [Biotin]$_{CBA}$ and [Biotin]$_{BA}$ indicate the biotin concentration measured by CBA and BA, respectively. $y=1.1x-0.26$, $r=0.997$.

Synthesis of Biotinylated IgA, IgM, and IgG with Different Biotin–Protein Ratios Two synthesis conditions were examined to prepare biotinylated IgA, IgM, and IgG with different biotin–protein ratios. First, the reaction time was varied. The results showed that it was also practically difficult to synthesize biotinylated IgA, IgM, and IgG with different biotin–protein ratios by varying the reaction time (data not shown).

Second, the ratio of NHS-biotin molecules to protein molecules was varied for synthesis of biotinylated proteins with different biotin–protein ratios. After synthesizing biotinylated proteins, the biotinylated proteins were freeze-dried and acid-hydrolyzed. The amount of biotin molecules released from biotinylated proteins after acid hydrolysis was measured by CBA. The amount of proteins was determined by protein assay kit and the biotin–protein ratio calculated.

The recoveries of biotin (5, 10, and 20 ng ml^{-1}) in this technique were 97±6, 102±8, and 101±4% ($n=3$), respectively.

The amount of released biotin increased with the increasing ratio of NHS-biotin molecules to protein molecules (data not shown). The biotin–protein ratios of biotinylated immunoglobulins (B/Ig)$_{af}$ were calculated and compared with the ratios of NHS-biotin molecules to immunoglobulin molecules (B/Ig) before synthesis of biotinylated immunoglobulins (Fig. 2). The results indicated that biotinylated immunoglobulins with a different biotin–protein ratio, (B/Ig)$_{af}$ were successfully created by varying the ratio of NHS-biotin molecules to immunoglobulin molecules instead of varying the reaction time.

The biotin–protein ratios of biotinylated IgA, IgM, IgG, and BSA were also determined using BA for biotin quantitation. The biotin–protein ratios of biotinylated IgA, IgM, IgG, and BSA measured by CBA and BA are shown in Table 1; the ratios were almost the same.

NHS-activated biotins react with primary amine groups in the side chain of lysine residues and the N-terminus of polypeptides. The number of lysine residues in BSA, IgA, IgM, and IgG summarized from the literature are shown in Table 1.[13—15] The biotin–protein ratio values obtained in this

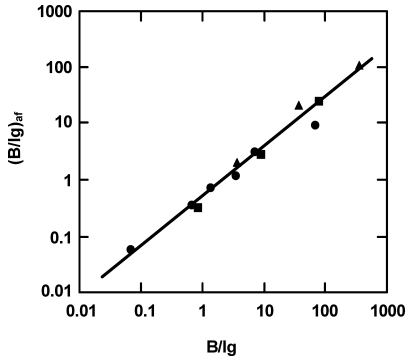

Fig. 2. Biotin–Protein Ratio, $(B/Ig)_{af}$ *vs.* the Ratio of NHS-Biotin Molecules to Immunoglobulin Molecules (B/Ig)

■, biotinylated IgA; ●, biotinylated IgG; ▲, biotinylated IgM. $r=0.989$.

Table 1. The Biotin–Protein Ratio of Biotinylated Proteins and the Number of Lysine Residues in the Proteins

Biotinylated proteins	Biotin–protein ratio		Proteins	Number of Lys residue*
	CBA	BA		
B-IgA	25±1.5**	22±2.5**	IgA	52—62
B-IgM	104±4.6**	94±8.3**	IgM	390—430
B-IgG	20±1.4**	19±1.5**	IgG	82—90
B-BSA	6.9	7.1	BSA	59

* The number of lysine residues was calculated from the data in References 13—15.
** $n=3$.

study were not higher than the number of lysine residues in the literature. None of the lysine residues in the proteins were biotinylated, indicating that only lysine residues on the surface of proteins were biotinylated whereas those inside the proteins were not biotinylated.

Stability of Biotinylated Proteins Biotinylated IgG in PBS or 1 mol l^{-1} HCl (control) were left at room temperature or 4, −20, or −80 °C for 0—30 d to investigate the stability of biotinylated IgG. The stability of freeze-dried biotinylated IgG stored at 4 °C, biotinylated BSA in PBS stored at −80 °C, and freeze-dried biotinylated BSA stored at 4 °C were also investigated. As shown in Fig. 3, biotinylated IgG in PBS stored at room temperature was stable for about two days, but then gradually released biotin from biotinylated IgG with time. Biotinylated IgG and BSA in PBS stored at 4, −20, or −80 °C were stable as were freeze-dried biotinylated IgG and BSA stored at 4 °C.

In this study, we successfully used a more direct technique, that is, biotinylated proteins were first acid-hydrolyzed and then the liberated biotin from the proteins was measured by

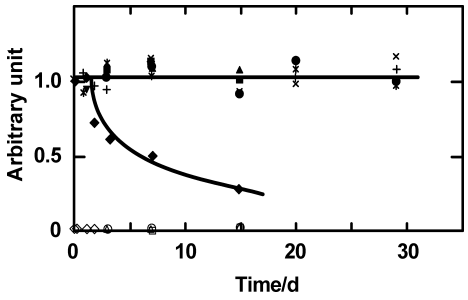

Fig. 3. Stability of Biotinylated Proteins Stored under Various Conditions

Biotinylated IgG in PBS: ◆, room temperature; ▲, 4 °C; ■, −20 °C; ●, −80 °C; ×, freeze-dried; biotinylated BSA in PBS: *, −80 °C; +, freeze-dried; biotinylated IgG in 1 mol l^{-1} HCl (control): ◇, room temperature; △, 4 °C; □, −20 °C; ○, −80 °C. An arbitrary unit decrease means a decrease in the stability of biotinylated proteins.

CBA or BA. The biotin–protein ratios of biotinylated IgA, IgM, and IgG, which were required as standards for biotin-binding immunoglobulins in human sera, were determined.

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