J Life Sci Res 2019; **17**: 7-10

Journal of Life Science Research
Online ISSN 2186-5809

Short Communication

Influence of the spacer arm length of biotinylation reagent for preparing a biotinylated microplate for biotin assay

Makoto Muratsugu † and Kouichi Nishida

Bioanalytical Science Laboratory, Department of Clinical Nutrition, Osaka Prefecture University, 3-7-30 Habikino, Habikino, Osaka 583-8555, Japan.

Received 4 March 2019; accepted 29 August 2019

We directly introduced biotin moieties into an amino group-rich microplate using biotinylation reagents. The biotinylated microplate could be successfully used for a solid phase-supported ligand competitive assay for biotin. The distance between the biotin-immobilized solid phase and the ureido ring of biotin was called the bridge length in this study. Examining biotinylation reagents with various spacer arm lengths led to a good dose-response curve as the bridge length increased. This result, supported by computer simulation, suggested that the steric hindrance of enzyme-labeled streptavidin affected its binding to immobilized biotin.

Key words: biotinylation reagent; biotinylated microplate; competitive assay; computer simulation

1 Introduction

Biotin is a water-soluble vitamin and a growth factor for microorganisms and animals,[1] and is widely distributed in foods.[2] A variety of methods for measuring biotin have been developed, and the biotin contents of natural materials have been determined. Many microorganisms that require biotin as a growth factor have been used for detecting biotin, and a turbidimetric method using *Lactobacillus plantarum* has been widely used.[3-5] An agar plate method using *L. plantarum* was also developed, offering greater precision than the turbidimetric method.[6]

Using the high affinity of avidin or streptavidin for the ureido ring of biotin, a solid phase-supported receptor competitive assay and a solid phase-supported ligand competitive assay have been developed as biotin assays.[7] In the former assay, biotin and enzyme-labeled biotin are added almost simultaneously to the avidin-immobilized solid phase on a microplate, and unbound biotin and labeled biotin are removed. In the latter assay, free biotin is first added to the biotin-immobilized solid phase, the enzyme-labeled avidin or streptavidin is immediately added, and the biotin-bound enzyme-labeled avidin or streptavidin is finally removed. To immobilize biotin on the solid phase, biotinylated proteins are generally adsorbed onto a microplate. For example, biotinylated bovine serum albumin[7-9] or goat anti-rabbit immunoglobulin G have been used as biotinylated proteins.[10]

In this study, we directly bound biotin to a microplate and investigated the influence of the spacer arm length of biotinylation reagent for preparing a biotinylated microplate.

2 Materials and Methods

2.1 Materials

The following materials were obtained from the sources indicated: flat-bottomed multiwell microplates (96 wells, Summilon A, Aminoplate) and ELISA Color Reagent kit (Type O) from Sumitomo Bakelite Co., Ltd., Tokyo, Japan; *N*-hydroxysuccinimidobiotin (NHS-biotin), sulfosuccinimidyl-6-biotinamido-hexanoate (Sulfo-NHS-LC-biotin), succinimidyl-6'-(biotinamido)-6-hexanamido hexanoate (NHS-LC-LC-biotin), and biotin pentafluorophenyl-ester (PFP-biotin) from Pierce Chemical Co., Rockford, IL, U.S.A. (Fig. 1); biotin, horseradish peroxidase (HRP)-conjugated streptavidin (HRP-streptavidin), bovine serum albumin (BSA), and dimethyl sulfoxide (DMSO) from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of reagent grade or better. The water used was 17-Mohm grade.

2.2 Biotinylated microplate

NHS-biotin, Sulfo-NHS-LC-biotin, NHS-LC-LC-biotin, and PFP-biotin were used to biotinylate Aminoplate, which is rich in amino groups on the surface of the plate. The biotinylation reagents were dissolved in DMSO, then diluted with 0.1 mol L^{-1} sodium phosphate buffer, pH 7.4, containing

† Corresponding author, Email: mmakoto@rehab.osakafu-u.ac.jp

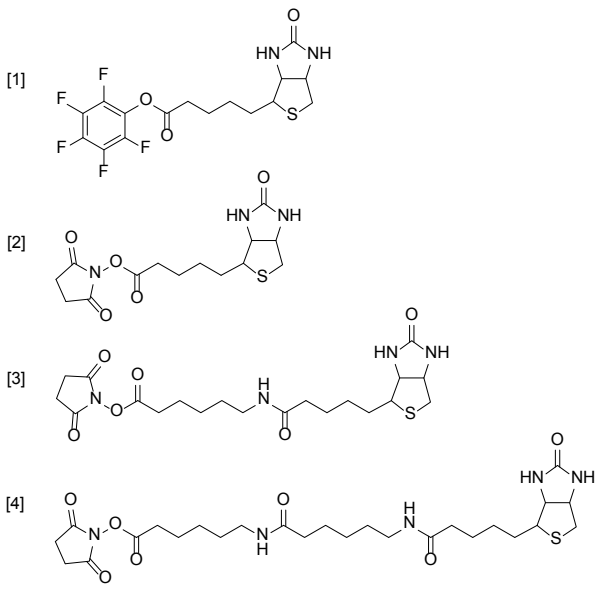

Fig. 1 Structure of biotinylation reagents.
[1] PFP-biotin (spacer arm length, 0.96 nm), [2] NHS-biotin (1.35 nm), [3] Sulfo-NHS-LC-biotin (2.24 nm), [4] NHS-LC-LC-biotin (3.05 nm).

0.15 mol L^{-1} NaCl. Sulfo-NHS-LC-biotin is soluble in aqueous solution, but was dissolved in DMSO for comparison. One hundred microliters of the biotinylation reagent solution (1.5×10^{-5} mol L^{-1}) was added to each well of Aminoplate and incubated for 2 hours at 37°C. After the wells were washed three times with Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS) containing 0.02% NaN_3 (PBS-N), 300 µL of 1% BSA in PBS-N was added to each well. The plates were incubated for 2 hours at 37°C and washed three times with PBS containing 0.05% merthiolate (PBS-M). The biotinylated Aminoplate was designated as a biotinylated microplate in this study.

2.3 Dose-response curves for a solid phase-supported competitive assay

Fifty microliters of dilutions of biotin was added to each well of a biotinylated microplate followed by 50 µL of 0.1 µg mL^{-1} HRP-streptavidin in PBS-M containing 0.1% BSA (PBS-MB). The plates were incubated for 1 hour at 37°C, then washed three times with PBS-M. One hundred microliters of substrate solution in the ELISA Color Reagent kit was added to each well. After the plate was incubated for 20 minutes at 25°C, the color reaction was terminated by the addition of a reaction stop solution in the kit to each well. Absorbance at 490 nm was then measured using a microplate reader (Model 450, Bio-Rad Labs., Richmond, U.S.A.) and the data were transferred to a computer using a custom-made program for data analysis.[11]

2.4 Binding of HRP-streptavidin on the biotinylated microplate

An increase in HRP-streptavidin binding indicates an increase of biotin uptake into a microplate, such that the HRP-streptavidin binding method can be used to evaluate biotin uptake. Fifty microliters of PBS-M was added to each well of a biotinylated microplate followed by 50 µL of 0.1 µg mL^{-1} HRP-streptavidin in PBS-MB. The plates were incubated for 1 hour at 37°C, then washed three times with PBS-M. One hundred microliters of substrate solution in the ELISA Color Reagent kit was added to each well. After the plate was incubated for 20 minutes at 25°C, the color reaction was terminated by the addition of a reaction stop solution in the kit to each well. Absorbance at 490 nm was then measured using the microplate reader and the data were transferred to a computer using a custom-made program for data analysis.[11]

3 Results and Discussion

Various long- and short-chain biotinylation reagents have been synthesized[12] and can now be commercially purchased. Aminoplates were biotinylated using biotinylation reagents with varying spacer arm lengths. The lengths of PFP-biotin, NHS-biotin, Sulfo-NHS-LC-biotin, and NHS-LC-LC-biotin are 0.96, 1.35, 2.24, and 3.05 nm, respectively. After these reagents reacted with amino groups on the solid phase, the distance between the solid phase and the ureido ring of biotin was designated as the bridge length in this study. When the bridge lengths for NHS-LC-LC-biotin, Sulfo-NHS-LC-biotin, NHS-biotin, and PFP-biotin are represented by BL_1, BL_2, BL_3, and BL_4, respectively, we have $BL_1 > BL_2 > BL_3 = BL_4$. The dose-response curves were obtained using these biotinylated microplates (Fig. 2). A better dose-response curve was obtained as the bridge length increased. In addition, the binding of HRP-streptavidin increased as the bridge length increased (Fig. 3). These results are explained below. A good dose-response curve is obtained with an increase in immobilized biotin in the solid phase-supported ligand competitive assay,[7] but this may not have been the case here. Since the amount of each biotinylation

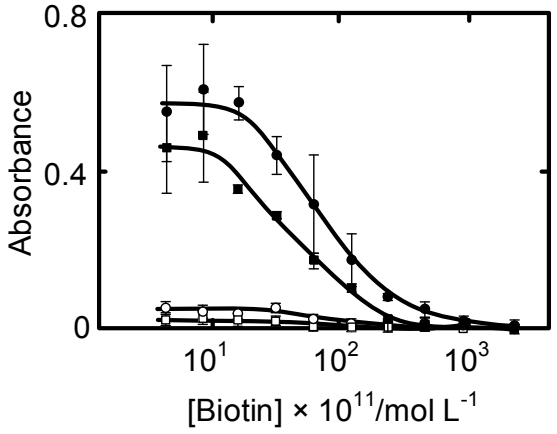

Fig. 2 Effect of the bridge length on dose-response curves.
The bridge length is defined in the text. □, 1.5×10^{-5} mol L^{-1} PFP-biotin; ○, 1.5×10^{-5} mol L^{-1} NHS-biotin; ■, 1.5×10^{-5} mol L^{-1} Sulfo-NHS-LC-biotin; ●, 1.5×10^{-5} mol L^{-1} NHS-LC-LC-biotin. 0.1 µg mL^{-1} HRP-streptavidin. Vertical bars indicate the standard deviation from the mean of duplicate experiments.

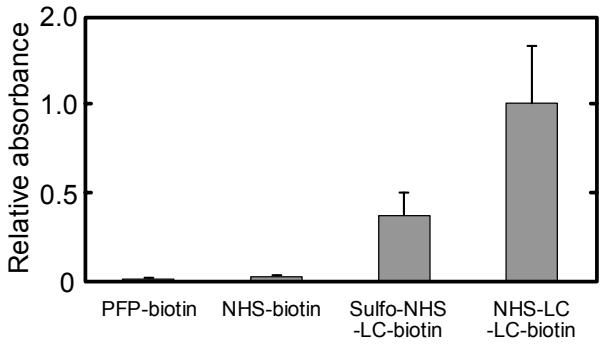

Fig. 3 Effect of the bridge length on HRP-streptavidin binding experiments.

1.5×10^{-5} mol L^{-1} PFP-biotin, NHS-biotin, Sulfo-NHS-LC-biotin, and NHS-LC-LC-biotin. 0.1 μg mL^{-1} HRP-streptavidin. Relative absorbance means that the absorbance for NHS-LC-LC-biotin is the standard value for comparison. Vertical bars indicate the standard deviation from the mean of duplicate experiments.

reagent used in this experiment was constant (1.5×10^{-5} mol L^{-1}), and these reagents are small molecules (the molecular masses of PFP-biotin, NHS-biotin, Sulfo-NHS-LC-biotin, and NHS-LC-LC-biotin are 410, 341, 557, and 568 Da, respectively), the collision frequency between these reagents and amino groups in the solid phase could possibly be the same; therefore, the number of immobilized biotin molecules appears to be the same irrespective of the biotinylation reagent used. In contrast, biotin is much smaller than HRP-streptavidin, the molecular masses of biotin, HRP, and streptavidin being 0.224, 440, and 680 kDa, respectively. The large HRP-streptavidin demonstrated steric hindrance of biotin binding (Fig. 4). When the bridge length is short, the number of HRP-streptavidin complexes bound to immobilized biotin may decrease (Fig. 4).

The idea that the steric hindrance of HRP-streptavidin affected its binding to immobilized biotin was confirmed by computer simulation using a mathematical model of the assay (Appendix). The steric hindrance of HRP-streptavidin is considered to correspond to a decrease in the biotin groups immobilized on the solid phase. We introduced an α coefficient ($0 < \alpha \leq 1$) into Eq. A6 in the Appendix, that is:

$$(1) \qquad ab_s = [B_s] + [B_s A^*].$$

Therefore, Eq. A8 is as follows,

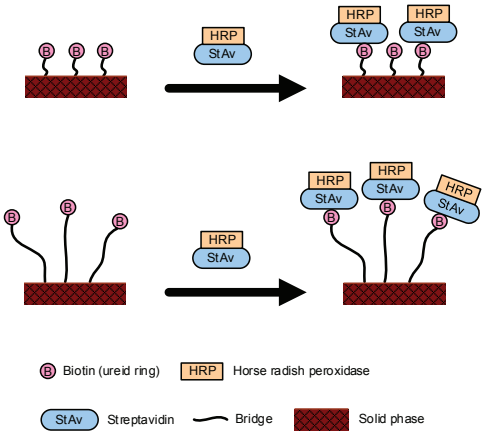

Fig. 4 Influence of the bridge length on the reaction of immobilized biotin and HRP-streptavidin.

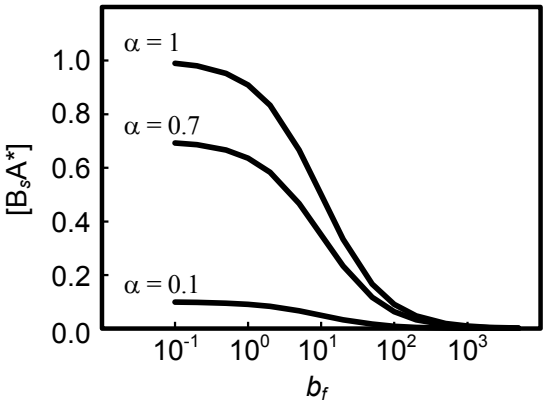

Fig. 5 Dose-response curves for the solid-phase-supported biotin competitive assay: effect of the α coefficient.

$K = 10^{15}$, $a^* = 1$, $b_s = 10$

small. The computer simulation is performed using Eq. 2 in Excel™ (Microsoft Co., Redmond, U.S.A.). Simulated plots of $[B_sA^*]$ as a function of b_f when changing the values of α are shown in Fig. 5. As α increases (decrease in steric hindrance of HRP-streptavidin), the response curves improve. The computer simulation supported the steric hindrance of HRP-streptavidin affecting its binding to immobilized biotin. In order to confirm this idea, it is necessary to experimentally compare the binding amount of a radioisotope labeled-HRP-streptavidin[13,14] on a biotin-bound microplate with a long bridge to its amount on the microplate with a short one. This is an issue for future research.

$$(2) \qquad [B_s A^*] = \frac{ab_s \left[\left[K(b_f + ab_s) + Ka^* + 1 \right] - \sqrt{K^2 (b_f + ab_s)^2 + 2K(1 - 2Ka^*)(b_f + ab_s) + (Ka^* + 1)^2} \right]}{2K(b_f + ab_s)},$$

where b_f represents the total concentration of biotin in standards or samples, and the intensity of color after an enzyme reaction is proportional to $[B_sA^*]$. As mentioned above, b_s is considered to be constant. If α is less than 1, it means that b_s is apparently

Conflict of Interest

All the authors declare that they have no conflicts of interest.

References

1 Wood HG (2008) Biotin, "The Vitamins: fundamental aspects in nutrition and health" 3rd ed. (Combs Jr GF, editor), Elsevier Academic Press, Burlington, pp. 331-344.

2 Mock DM (2001) Biotin, "Handbook of vitamins" 3rd ed. (Suttie JW, McCormick DB, Rucker RB, Machlin LJ, editors), CRC Press, New York, pp. 397-426.

3 Wright LD, Skeggs HR (1994) Determination of biotin with *Lactobacillus arabinosus*. Proc Soc Exp Biol Med, 56:95-98.

4 Manaka Y, Watanabe T (1990) Determination of biotin in blood. Vitamins, 64: 307-312 (in Japanese).

5 Ball GFM (2006) Microbiological methods for the determination of the B-group vitamins. "Vitamins in foods", Talor & Francis, Boca Raton, pp. 339-368.

6 Fukui T, Iinuma K, Oizumi J, Izumi Y (1994) Agar plate method using *Lactobacillus plantarum* for biotin determination in serum and urine. J Nutr Sci Vitaminol, 40:491-498.

7 Muratsugu M, Amano N, Takeda R (2009) Comparison of the solid phase-supported receptor and ligand competitive assays for biotin. J Health Sci, 55:939-945.

8 Bayer EA, Ben-Hur H, Wilchek M (1986) A sensitive enzyme assay for biotin, avidin, and streptavidin. Anal Biochem, 154:367-370.

9 Huang EZ (1997) Competitive enzymatic assay of biotin. Methods Enzymol, 279:304-308.

10 Shiuan D, Wu C-H, Chang Y-S, Chang, R-J (1997) Competitive enzyme-linked immunosorbent assay for biotin. Methods Enzymol, 279:321-326.

11 Yazawa A, Takeda R, Muratsugu M (2004) A simple computer program for the microplate reader. J Rehabil Health Sci, 2:28-32 (in Japanese).

12 Wilchek M, Baye, EA (1990) Biotin-containing reagents. Methods Enzymol, 184:123-138.

13 Muratsugu M, Kurosawa S, Kamo N (1991) Adsorption and desorption of $F(ab')_2$anti-hIgG on plasma-polymerized allylamine thin film: The application of the film immunoassay. J Colloid Interface Sci, 147:378-386.

14 Muratsugu M, Ohta F, Miya Y, Hosokawa T, Kurosawa S, Kamo N, and Ikeda H (1993) Quartz crystal microbalance for the detection of microgram quantities of human serum albumin: Relationship between the frequency change and the mass of protein adsorbed. Anal Chem, 65:2933-2937.

Appendix

Biotin binds strongly to streptavidin, which has four binding sites. We assumed that each biotin molecule (ligand) bound to a streptavidin molecule (receptor) in order to simplify the model. The reaction of the solid phase-supported ligand competitive assay for biotin[7] can be schematized in the following reaction schemes:

$$\text{(A1)} \quad B_f + A^* \rightleftharpoons B_f A^*$$

$$\text{(A2)} \quad B_s + A^* \rightleftharpoons B_s A^*$$

where B_f, B_s, and A* represent free biotin, solid phase-supported biotin, and enzyme-labeled streptavidin, respectively. The equilibrium constants for these reactions, schemes A1 and A2, are designated as K_f and K_s, respectively:

$$\text{(A3)} \quad K_f = \frac{[B_f A^*]}{[B_f]\,[A^*]}$$

$$\text{(A4)} \quad K_s = \frac{[B_s A^*]}{[B_s]\,[A^*]}$$

The total concentrations of free biotin (b_f), solid phase-supported biotin (b_s), and enzyme-labeled streptavidin (a^*) are given by

$$\text{(A5)} \quad b_f = [B_f] + [B_f A^*]$$

$$\text{(A6)} \quad b_s = [B_s] + [B_s A^*]$$

$$\text{(A7)} \quad a^* = [A^*] + [B_f A^*] + [B_s A^*]$$

Assuming $K_s = K_f (\equiv K)$, the following equation is derived from Eq. A3 to Eq. A7:

$$\text{(A8)} \quad [B_s A^*] = \frac{b_s \left[[K(b_f + b_s) + Ka^* + 1] \pm \sqrt{K^2 (b_f + b_s)^2 + 2K(1 - 2Ka^*)(b_f + b_s) + (Ka^* + 1)^2} \right]}{2K(b_f + ab_s)}.$$