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LUO, Shiwen
Department of Pharmacology, Faculty of Medicine, Saga Medical School

TAKANO, Masako
Department of Pharmacology, Faculty of Medicine, Saga Medical School

TAKASAKI, Yozo
Chemistry Laboratory, Faculty of Medicine, Saga Medical School

ASAKAWA, Takeo
Department of Pharmacology, Faculty of Medicine, Saga Medical School

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原 著

Measurement of Low Level Membrane Proteins Using Bicinchoninic Acid: Modified Procedures to Eliminate Interfering Substances

Shiwen LUO[1)†], Masako TAKANO[1)†], Yozo TAKASAKI[2)] and Takeo ASAKAWA[1)]

[1)]*Department of Pharmacology and* [2)]*Chemistry Laboratory, Faculty of Medicine, Saga Medical School, 5-1-1 Nabeshima, Saga 849-8501, Japan*

Abstract Accurate determination of low levels of protein in samples containing large amounts of interfering substances is rather difficult. Precipitation-bicinchoninic acid (BCA) assay as reported can concentrate and partially decontaminate the protein by adding sodium deoxycholate (DOC) and trichloroacetic acid (TCA) to the sample. Yet, this procedure alone has been insufficient for analysis of highly contaminated samples. In this report, we describe an improved method, the DOC-TCA-washing-BCA method, which is composed of the DOC-TCA precipitation and a subsequent aqueous washing that eliminates many interfering substances. The protein concentrations in samples containing even large amounts of interfering substances (e.g. various sugars and some detergents) were well quantitated by this method. The modifications described here have enabled us to perform rapid and efficient removal of many interfering substances that are commonly used in protein purification, and to allow proteins to be detected above 0.05 μg and from a solution above 0.5 μg/ml.

Key words: Protein measurement, Membrane protein, Bicinchoninic acid, Interfering substances

Introduction

Accurate determination of protein concentration is fundamental for all quantitative discussion of biochemical interactions. Only a limited number of assays are available for the quantitation of proteins in high dilutions[2)8)11)12)15)]. In practice, the quantitative determination of dilute protein samples, especially membrane-protein containing detergents like Triton X-100, phospholipids, reducing and non-reducing sugars, $MnCl_2$ or sulfhydryl compounds such as β-mercaptoethanol and dithiothreitol (DTT) is laborious because these substances interfere with the analysis. Several procedures that circumvent these problems by acid precipitation and/or precipitation by acetone or methanol-chloroform have been published[1)3)16)]. However, it is often difficult to find optimal precipitation conditions especially when working with hydrophobic (membrane) proteins in the presence of detergents and phospholipids. Protein quantitation based on bicinchoninic acid (BCA) is simple, sensitive and tolerant to many detergents and substances that are known to interfere with the Lowry method[12)]. The micro BCA protein assay

†These authors contributed equally to this work.

Corresponding author: Takeo Asakawa M.D. & Ph.D.

reagent is highly sensitive for the quantitative colorimetric determination of total protein in dilute aqueous solutions. Color development in the micro BCA protein assay is performed at 60°C rather than 37°C because this change reduces protein-to-protein variability and doubles sensitivity[12)17)]. However, other substances such as phospholipids[5)] and ammonium sulfate[3)], either naturally present in the sample or added during protein purification, have been shown to still interfere with the BCA protein assay. Since the assay involves reduction of Cu^{2+} to Cu^{+} by proteins, reducing compounds (such as DTT, cysteine or reducing sugars) or copper-chelating reagents (such as EDTA) cause interference[4)6)]. Furthermore, the assay does not tolerate solutions that change the pH of the working reagent[12)]. Interference caused by DTT, reducing sugars, EDTA, ammonium sulfate, etc. can be eliminated to some extent by precipitating proteins selectively with sodium deoxycholate (DOC) and trichloroacetic acid (TCA) prior to reaction with BCA[3)]. While employing the DOC-TCA precipitation BCA protein assay to quantitate protein, we noted that certain interfering contaminants that remained in the protein precipitate (e.g., phospholipids, reducing sugars) strongly affected the color development of the BCA chromophore. The interference observed is particularly troublesome when comparing protein levels at various steps of protein purification, for different kinds of buffer components are present in each step. By introducing an aqueous washing modification to the DOC-TCA precipitation BCA protein assay we successfully removed these interfering agents. The data in this paper show that the modified procedure is compatible with protein quantitation using the micro BCA protein assay reagent and is useful for removing a wide variety of interfering substances.

Materials and Methods

Materials

Micro BCA protein assay reagent kit was purchased from Pierce (Rockford, IL). Bovine serum albumin, Lubrol-PX, Triton X-100, octyl β-D-glucoside, phosphatidylcholine, Hepes, methyl α-D-mannoside (MeMan), methyl α-D-glucoside (MeGlu), N-acetylgalactosamine (GalNAc), L-fucose (Fuc), D-galactose (Gal), α-lactose (Lac) and N-acetylglucosamine (GlcNAc) were obtained from Sigma (St.Louis, MO). Sodium deoxycholate and TCA were obtained from Wako Pure Chemical Industries (Osaka), and DTT from Nacalai Tesque (Kyoto). Other reagents were analytical grade. Actual protein concentrations were determined spectrophotometrically using bovine serum albumin (BSA) as a standard, and applying an extinction coefficient of 6.6 (1% solution at 280 nm)[13)].

Recommended Procedure

In this study, we employed 6 X 50-mm disposable borosilicate glass culture tubes (KIMBLE, No. 73500-650) for the assay. The modified DOC-TCA precipitation BCA protein assay, the novel method reported here, was developed from the original method of Brown et al.[3)] by incorporating several critical modifications. The assay's working reagent was prepared by mixing 25 vol of micro BCA Reagent A (Na_2CO_3, $NaHCO_3$ and Na-tartrate in 0.2 N NaOH), 24 vol of Reagent B (4% BCA) and 1 vol of Reagent C (4% $CuSO_4$. $5H_2O$) as described by the supplier. The procedures described below were carried out at room temperature (20°C). To solutions containing 0.05 to 3.0

μg protein in 0.1 ml, 20 μl (20～200 μl, see Fig. 1) of DOC (0.15%, w/v) was added. After standing for 20 min, 10 μl (10～25 μl, see Fig. 1) of TCA (72%, w/v) was added to give a final concentration of 5.5%. The samples were incubated for at least 3～7 min, then centrifuged for 15 min at 9000 X g at 15°C to precipitate protein. The supernatant fluid was removed by aspiration with a Pasteur pipet connected to a water-suction device. To remove interfering substances, the following washing step had to

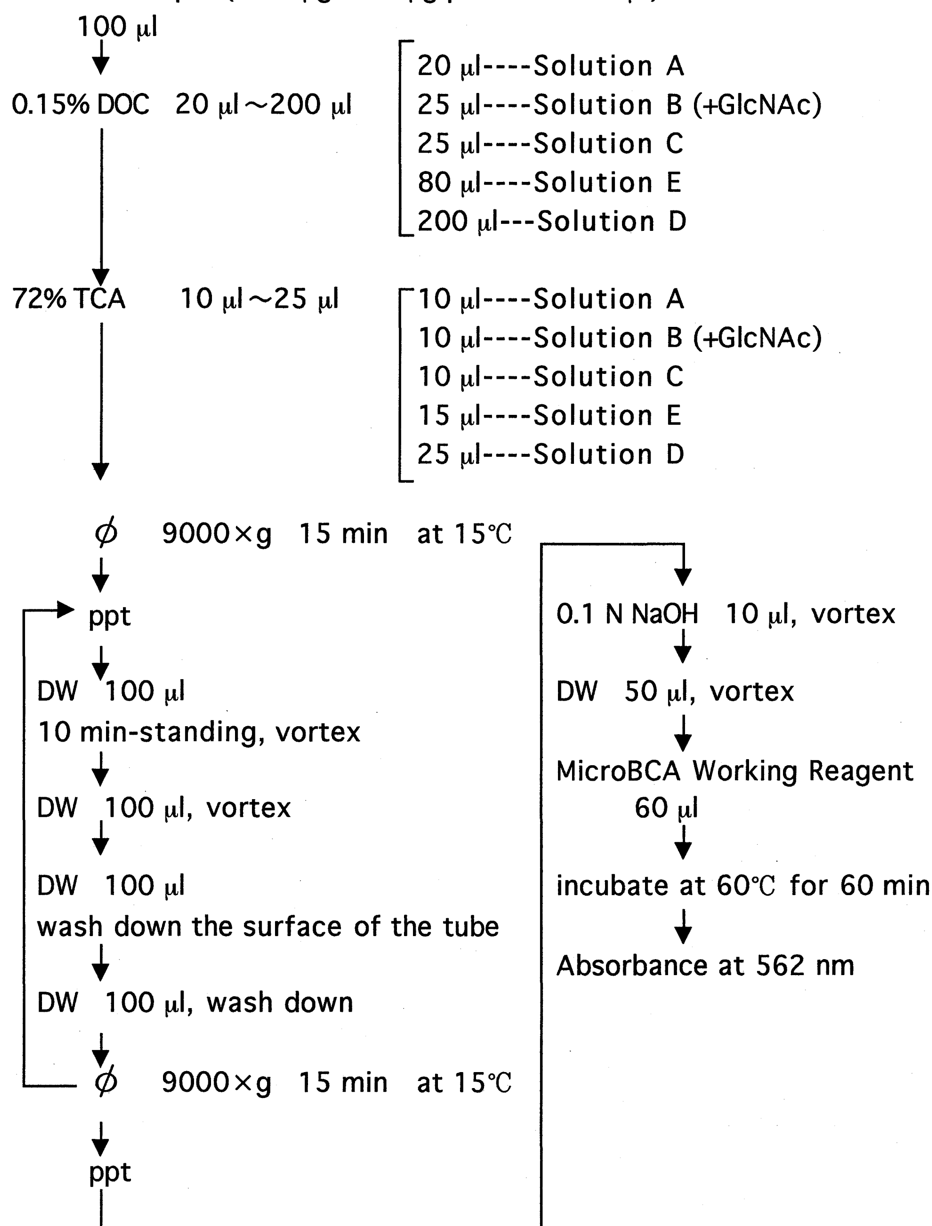

Fig. 1 Scheme of the DOC-TCA-washing-BCA method.
Experimental conditions are described under Materials and Methods. DW: distilled water. The components of solutions A～E are described under Materials and Methods.

be repeated twice. Pellets were suspended stepwise with 2 portions of 100 μl distilled water by vortex, and subsequently the inside surface of the tubes was washed down without vortex with 2 portions of 100 μl distilled water. Then, the suspensions were centrifuged for 15 min at 9000 X g. The supernatants were carefully discarded by aspiration. The pellets obtained with the repeated washing were dissolved in 10 μl of 0.1 N NaOH by vigorous mixing. Then 50 μl of distilled water and 60 μl of BCA working reagent were immediately sequentially added. Each tube was vortexed, sealed and incubated for 60 min at 60μC. The tubes were allowed to cool to room temperature, and the optical density at 562 nm was promptly measured by a Beckman DU-65 spectrophotometer, against water. Proper centrifugation and aspiration are essential to achieve quantitative recovery of the protein. All the assays were carried out in duplicate.

Interfering Substances

Solutions containing interfering substances were as follows:

solution A: 20 mM Tris-HCl, 0.1% Lubrol-PX, 0.025% phosphatidylcholine, 20% glycerol, 2 mM $MnCl_2$, 1 mM NaN_3, 0.1 mM PMSF, pH 7.4

solution B: 20 mM Hepes, 0.1% Lubrol-PX, 0.025% phosphatidylcholine, 20% glycerol, 1 mM EDTA, 0.5 M NaCl, 0.1 mM PMSF, pH 7.4

solution C: 20 mM Tris-HCl, 0.15% Triton X-100, 0.5 M NaCl, 0.025% phosphatidylcholine, 2 mM DTT, pH 7.4

solution D: 20 mM Tris-HCl, 1% Triton X-100, 0.5 M NaCl, 2 mM DTT, pH 7.4

solution E: 20 mM Tris-HCl, 30 mM octyl β-D-glucoside, 0.5 M NaCl, 2 mM DTT, pH 7.4

The reasons for dissolving BSA in the above solutions, which were then used to mimic complex samples, were that: (1) phosphatidylcholine, glycerol, PMSF, NaN_3 and detergents such as octyl β-D-glucoside, Lubrol-PX and Triton X-100, are common reagents used in membrane protein research, (2) when performing lectin affinity chromatography, the protein to be assayed is often present in a solution of reducing sugar. These mimic complexes could be expected to produce hard test conditions for quantitative precipitation of protein and the elimination of the interfering substances. For the purpose of comparing the modified DOC-TCA precipitation BCA protein assay with the classical assay, a sample size of 0.1 ml containing 0.05 to 3.0 μg of BSA was chosen. Fig. 1 summarizes our procedures for protein determination. We named this improved method, DOC-TCA- washing-BCA method.

Results and Discussion

1. Modification of the Original DOC-TCA Precipitation Micro BCA Protein Assay

We previously found that in the conventional micro BCA protein assay (i.e. without precipitation nor aqueous washing step), the optical density obtained at 562 nm was >3 in the presence of interfering substances, which makes any protein determination impossible. To negate the deleterious effects of different interfering agents, protein precipitation was performed prior to BCA

assay by Brown et al.[3]. However, reducing sugars like N-acetylglucosamine, phospholipids and sulfhydryl compounds like DTT caused intense color development in the BCA assay particularly when the assay was run at higher temperatures to maximize sensitivity[4)5)12)].

We have worked on membrane proteins, carrying out lectin affinity chromatography using various kinds of sugar derivatives. In such study, exact protein determination is necessary and we have wasted many kinds of samples for this reason. Table 1 shows the effect of two times DOC-TCA treatment on the conventional BCA assay in the presence of various kinds of sugars. Without any treatment, O.D. at 562 nm of distilled water and BSA (0.4 μg) were 0.182 and 0.488, respectively. Addition of sugars yielded a remarkable increase in absorbance, and there was little difference in absorbance between a solvent and a BSA solution.

These results were again obtained when two times treatment with DOC and TCA were performed. Absorbances were still high, and significant BSA measurement was impossible.

To eliminate these interferences and maximize the resultant color yield of protein, several different approaches have been undertaken. For example, SDS was subsequently added for minimizing interferences by lipids and to promote rapid solubilization of the precipitate as suggested by Peterson[9] and Morton et al.[7]. Nevertheless, high temperature and prolonged exposure to alkali caused serious oxidation of lipids. Another procedure[14] designed to remove interfering lipids by extraction of TCA-precipitated samples with organic solvents such as diethyl ether was also examined.

We found though that the aqueous wash-

Table 1 Effect of a two times DOC-TCA treatment on the conventional micro BCA method in the presence of sugars

Sample	Absorbance at 562 nm			
	1st DOC-TCA		2nd DOC-TCA	
	–	0.4 μg BSA	–	0.4 μg BSA
Control	0.178	0.453	–	–
Solution A	2.27	2.08	2.20	2.30
Solution B	2.50	2.47	2.26	2.38
+ 0.25 M GlcNAc	> 6	> 6	2.19	2.47
+ 0.2 M GalNAc	> 6	> 6	2.27	2.43
+ 0.2 M Gal	> 6	> 6	2.27	2.29
+ 0.2 M Lac	> 6	> 6	2.23	2.40
+ 20 mM Fuc	2.69	2.96	2.23	2.37
+ 0.5 M MeMan	2.35	2.69	2.25	2.38
+ 0.5 M MeGlu	2.20	2.53	2.21	2.38

Solution A: 20 mM Tris-HCl, 0.1% Lubrol-PX, 20% glycerol, 0.025% phosphatidylcholine, 2 mM $MnCl_2$, 1 mM NaN_3, 0.1 mM PMSF

Solution B: 20 mM Hepes, 0.1% Lubrol-PX, 20% glycerol, 0.025% phosphatidylcholine, 0.5 M NaCl, 1 mM EDTA, 0.1 mM PMSF

BSA (each 0.4 μg) was treated with DOC and TCA then centrifuged to give a pellet which was suspended in distilled water and again precipitated with DOC and TCA, then the eventual precipitate was subjected to micro BCA assay, as described under Materials and Methods. –, no BSA.

ing step removed various kinds of interfering compounds, in a satisfactory way, from the protein precipitate. We have confirmed that the repeated aqueous washing with 2-times- or even with 3-times-exchanges of water does not reduce amount of precipitated proteins at all. Fig. 1 illustrates the scheme of the DOC-TCA washing BCA method.

2. Linearity, Sensitivity and Reproducibility of the Modified Procedure

Fig. 2 illustrates that a linear standard curve is adaptable in the range of 0.05 to 1.0 μg of BSA in solutions A and B, which contain interfering substances. The slopes of the curves are similar to a conventional standard curve obtained with an untreated BSA control sample. The two curves, however, differed from the control by a constant value of 0.07~0.1 absorbance units in the range from 0.1 to 1.0 μg protein, possibly due to residual interfering substances. Similar analysis using solution D or E containing different amounts of BSA yielded a straight line (Fig. 3). Also, it is noteworthy that the residual interfering substances caused little change in the observed slope values in the range of 0.1 to 1.0 μg of BSA. For example, in the absence of interfering substances, by untreated analysis, 0.05 to 1.0 μg of BSA resulted in a mean slope value of 0.698 ± 0.024 O.D. units per μg of BSA (n=14). While with BSA in solution A, the mean slope value was 0.697 ± 0.033 units per μg of BSA (n=24) by this modified procedure. Moreover, the low standard deviation values indicated the high degree

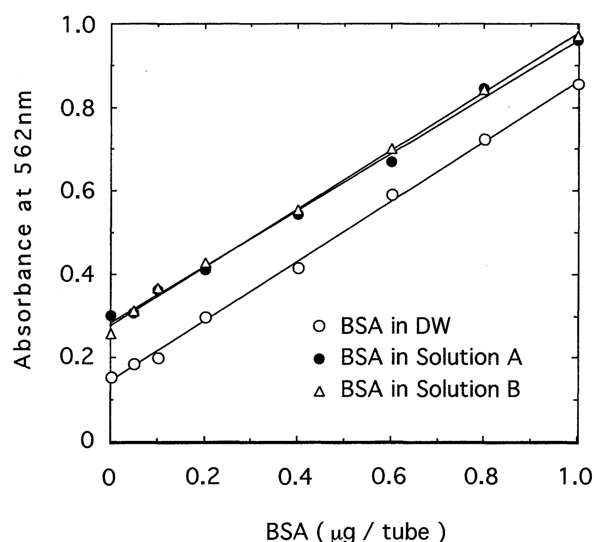

Fig. 2 The standard concentration curves with various amounts of BSA in solutions A and B. Various amounts of BSA were dissolved in solution A (closed circles) or in solution B (open triangles) and applied to the procedures for the DOC-TCA-washing-BCA method. As a control, micro BCA protein assay was directly performed on BSA dissolved in distilled water (DW, open circles). Data represent a mean of duplicate estimations.

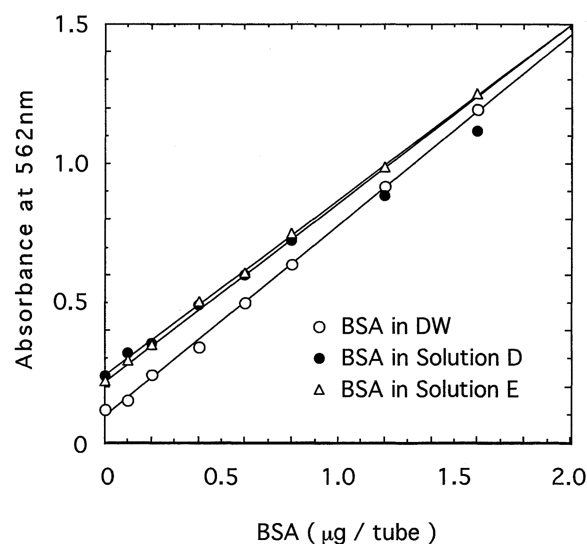

Fig. 3 The standard concentration curves with BSA in the presence of detergents. Various amounts of BSA were dissolved in solution D containing 1% Triton X-100 (closed circles) and solution E containing 30 mM octyl β-D-glucoside (open triangles) and applied to the procedures for the DOC-TCA-washing-BCA method. As a control, micro BCA protein assay was directly performed on BSA dissolved in distilled water (DW, open circles). Data represent a mean of duplicate estimations.

of reproducibility that we observed with this assay. Furthermore, comparison with control experiments using BSA indicated that the DOC-TCA-washing-BCA method provided a quantitative recovery of protein.

3. Elimination of Interfering Effects of Sugars and Detergents

Fig. 4 shows that in the presence of a number of interfering substances including reducing or non-reducing sugars, BSA (0.2 ~0.6 μg/0.1 ml) determination can be done by the DOC-TCA-washing-BCA method without an increase in absorbance due to residues of sugars of more than 2% of the control without sugar. Further, the slope and intersection of the lines are almost constant independently of sugars.

Nonionic detergents such as Triton X-100

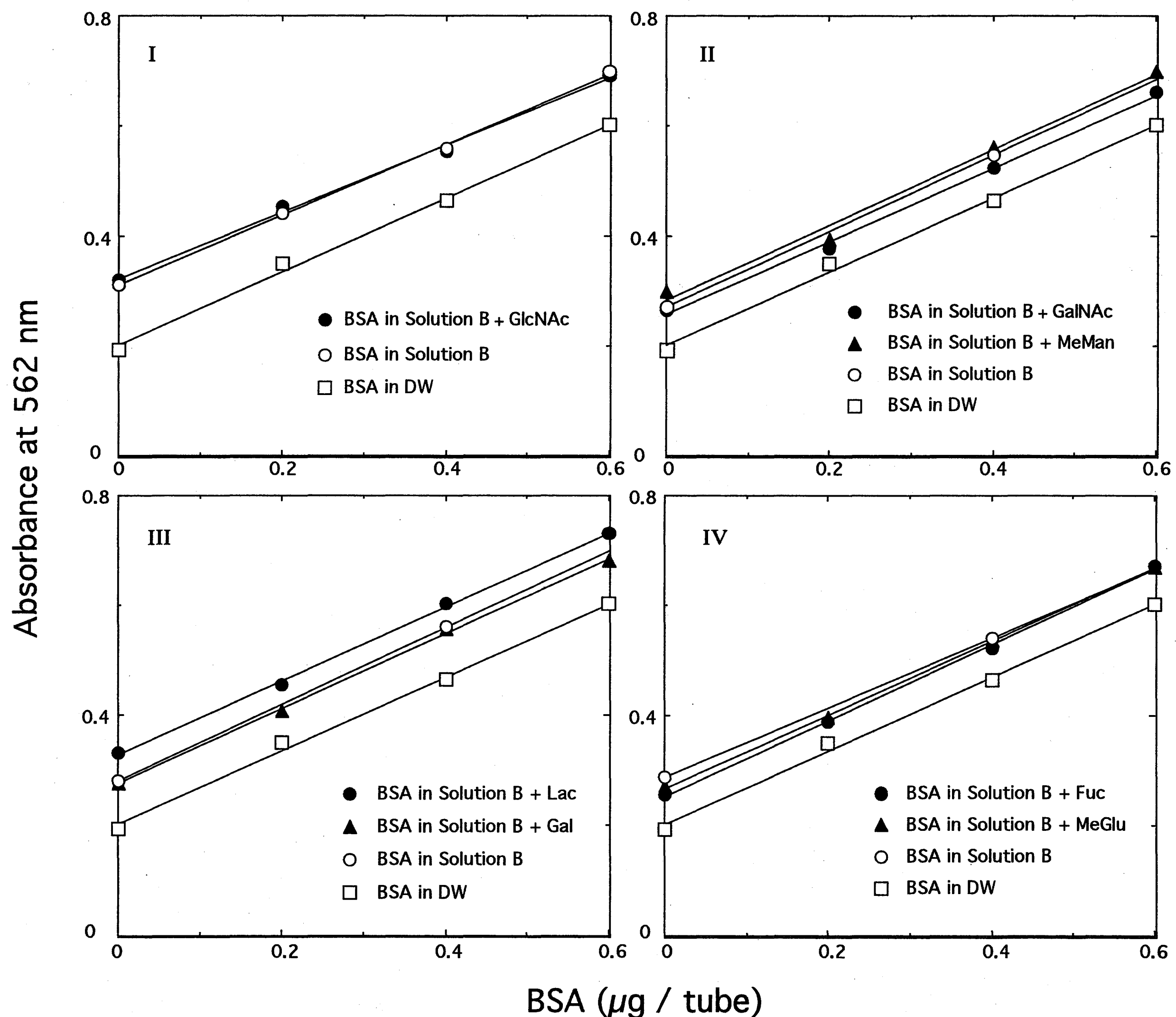

Fig. 4 The standard concentration curves with BSA in solution B containing sugars used in a lectin affinity chromatography. Various amounts of BSA were dissolved in solution B containing sugars, as follows; in (I), GlcNAc, 0.25 M N-acetylglucosamine (closed circles); in (II), GalNAc, 0.2 M N-acetylgalactosamine (closed circles), and MeMan, 0.5 M methyl α-D-mannoside (closed triangles); in (III), Lac, 0.2 M α-lactose (closed circles), and Gal, 0.2 M D-galactose (closed triangles); in (IV), Fuc, 20 mM L-fucose (closed circles) and MeGlu, 0.5 M methyl α-D-glucoside (closed triangles). As a control, micro BCA protein assay was directly performed on BSA dissolved in distilled water (DW, open squares). Data represent a mean of duplicate estimations.

and octyl β-D-glucoside are often used in membrane bound protein preparation. However, such detergents prevent a quantitative precipitation of protein, as has been reported[10]. It was originally reported[3] that a final DOC concentration of 125 μg/ml gave optimal precipitation with a number of proteins. The amounts of DOC or TCA solution required for a quantitative precipitation of protein depended on the sort of detergents and their concentrations contained in a protein solution. Then, we determined what amounts of DOC and TCA were required for getting a quantitative precipitation. As shown in Fig. 1, 0.1 ml of solution B and C or 0.1 ml of solution D required 25 μl or 200 μl of DOC solution (0.15% solution), respectively, and a volume of TCA (72% solution) required was 10 μl for solution B and C and 25 μl for solution D. In the case of solution E, 80 μl of DOC (0.15% solution) and 15 μl of TCA were required for obtaining a quantitative precipitation of protein (Fig. 1 and 3).

4. Estimation of Membrane Protein

The DOC-TCA-washing-BCA method described in this paper, gave a linear standard curve with various amounts of BSA that were dissolved in a sample-buffer solution containing interfering substances. From this standard BSA curve, protein values of the membrane-protein samples in the sample-buffer solution containing various interfering substances were determined by the DOC-TCA-washing-BCA method. Proteins of isolated rat lung membranes were employed as the simulation samples in Fig. 5. Lung membranes were solubilized with 30 mM octyl β-D-glucoside and loaded on GTP-agarose column. The eluate fraction from GTP-agarose was diluted with solution A to 4 μg protein/ml and used as a simulation sample, which was applied to procedures for the DOC-TCA-washing-BCA method. As shown in Fig. 5, two different volumes (e.g., 60 μl and 100 μl) of aliquots from each sample were applied to the assay procedures. From absorption at

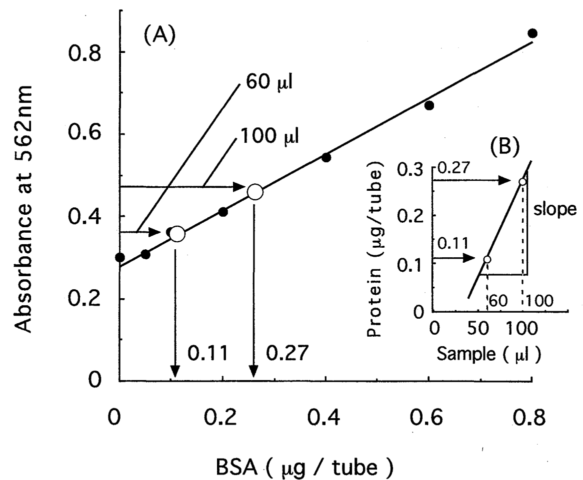

Fig. 5 Estimation of membrane protein in solution A (an eluate fraction of GTP-agarose as a simulation sample).
(A) The standard concentration curve with BSA in solution A. A simulation sample (4 μg protein/ml) was applied to procedures for the DOC-TCA-washing-BCA method. In this simulation, 60 μl and 100 μl of aliquots from the sample were applied to the assay procedures. From absorption at 562 nm, the value of protein in 60 μl or 100 μl sample on the standard concentration curve was read.
(B) The values of protein in a sample were plotted against the initial volumes of the sample applied to the assay. The concentration of protein of the simulation samples was obtained from the slope (0.41 μg/0.1 ml). The simulation sample was obtained as follows; lung membranes were solubilized with 30 mM octyl β-D-glucoside and loaded on GTP-agarose column. The eluate fraction from GTP-agarose was diluted with solution A to 4 μg protein/ml and used as a simulation sample.

562 nm, the value of protein in the 60 μl or 100 μl sample on the standard concentration curve with BSA in solution A as standard (Fig. 5A) was read, and then the values of protein in the sample were plotted against the initial volumes of the sample applied to the assay. The concentrations of the proteins of the simulation samples were obtained from the slope (Fig. 5B). At least, two different volumes of aliquots from each sample needed to be applied to the assay procedures in order to get the slope in plotting the amount of protein vs. volume of sample.

In summary, the DOC-TCA-washing-BCA method depends on the quantitative precipitation of protein while interfering substances are washed away by a brief aqueous washing step. The procedure described here is quite effective in eliminating the interference in BCA protein assay caused by detergents, sulfhydryl compounds, reducing sugars, phosphatidylcholine, EDTA, $MnCl_2$, etc. However, because the aqueous washings of samples to remove interfering substances have to be done individually, a possible disadvantage of the method is that variations in OD_{256} values obtained due to a trace amount of residual interfering substances are somewhat greater than that in the conventional assay.

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（和文抄録）

Bicinchoninic acid（BCA）法による膜タンパク質の微量定量 ― 妨害物質の影響を除去する方法の考案

[1]佐賀医科大学医学部薬理学講座，[2]佐賀医科大学医学部化学

Shiwen Luo[1]，高野正子[1]，高崎洋三[2]，麻川武雄[1]

一般に膜結合型の活性蛋白質の抽出および精製において，デタジェントをはじめ安定化のために buffer 系に添加される物質は，多くの蛋白質定量法に対して，妨害物質となる場合が多い．我々は DOC-TCA 法に引き続いて，水で washing を行うことにより，DOC-TCA 法では除去できない妨害物質（デタジェント，グリセロール，リン脂質，Mn^{2+}，DTT，及びレクチン親和クロマトグラフィーに用いられる糖誘導体など）の影響をほぼ完全に除去することができた．即ち DOC-TCA-washing の後，BCA（bicinchoninic acid）反応により，0.05 μg～1.6 μg の微量の蛋白質を感度良く，正確に，再現性良く，定量することができる．