Determination of biotinylated proteins as an index for purification of plasma membrane using surface plasmon resonance-based optical biosensor

Shiro Okumura^{1,*}, Tetsuyuki Akao¹, Satoko Yamashita¹, Tokio Ichimatsu¹ and Kuniyo Inouye²

¹Fukuoka Industrial Technology Center, 1465-5 Aikawa, Kurume, Fukuoka 839-0861, Japan; ²Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan; *Author for correspondence (e-mail: sokumura@fitc.pref.fukuoka.jp; phone: +81-942-30-6644; fax: +81-942-30-7244)

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Abstract

Proteins of plasma membrane could be an index of purification of the plasma membrane of animal cells. A convenient method is proposed for determining the plasma membrane proteins by a surface plasmon resonance (SPR) biosensor. Biotinylated proteins were observed only in the peripheral areas of MOLT-4 cells which were treated by 5-[5-(*N*-succinimidylloxycarbonyl) pentylamido] hexyl-D-biotinamide. The proteins on HeLa cells were also biotinylated. And then the membrane samples of the HeLa cells were injected onto the avidin-immobilized SPR-surface, and components bound non-specifically on the surface were removed by a washout solution. The amount of biotinylated protein (BP) was determined directly from the absolute resonance unit (RU) after injection of the washout solution. In the method a reference surface was not needed. The amount of BP bound to the surface was gradually attenuated with the repeated injection, and a method for calibrating the RU value was introduced by considering the ratio of attenuation by every injection. The correlation between the BP titer calculated by the calibration and the theoretically-estimated one was greatly improved. Three cycles of the BP determination on a sensor surface was performed successfully. During the purification process of membrane fractions, the degree of purification as judged by the BP titer was in good agreement with the degree of increase in aminopeptidase N activity in the membrane fraction. Thus, the BP titer could be used as an index for purification of plasma membrane.

Abbreviations: AEBSF – 4-(2-aminoethyl)-benzenesulfonyl fluoride; BP – biotinylated protein(s); BSA – bovine serum albumin; DMSO – dimethyl sulfoxide; E-64 – trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane; EDC – *N*-ethyl-*N'*-(3-diethylamino-propyl) carbodiimide; GTA – 3,3-dimethylglutaric acid; HBS – HEPES-buffered saline; HEPES – 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MEM – minimum essential medium; NHS – *N*-hydroxysuccinimide; OVA – ovalbumin; PBS – phosphate-buffered saline; RU – resonance unit; SD – standard deviation; SPR – surface plasmon resonance

Introduction

Plasma membrane is generally purified according to measuring the activities of marker enzymes as a

purification index. For example, alkaline phosphatase, Mg²⁺-ATPase, and 5'-nucleotidase are used as marker enzymes of plasma membrane, while acid phosphatase and glucose 6-phosphatase are as

marker enzymes of membrane fractions of lysosome and endoplasmic reticulum, respectively (Lin et al. 1987). However, a universal index has been needed for plasma membrane purification of any cell line. The objective of the present study is to develop a method to determine BP as an index of plasma membrane purification.

Biotinylation of proteins (Hurley et al. 1985; Cole et al. 1987) can be applied in a variety of purposes such as immunodetection on western blots, immunoprecipitation, and ligand blotting (Meier et al. 1992; Nesbitt and Horton 1992; Inostroza et al. 1992). Many biotinylation reagents are provided to form covalent bonds with an amino group or a thiol group of proteins or a hydroxyl group of reducing sugar. Because biotin molecules are introduced to proteins only on plasmalemma when the cells are treated with biotinylation reagents (Meier et al. 1992), the ratio of BP to total proteins may be used for an index of plasma membrane purification. In the present study, we describe that BP can be determined by SPR-based optical biosensor and that this method could provide us with an index in purifying membrane fractions from various cells.

Materials and methods

Instrumentation and reagents

The SPR detection system, Biacore 1000, and CM5 sensor chips and amine coupling kit were obtained from Biacore AB (Uppsala, Sweden). 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was from Dojindo Laboratories (Kumamoto, Japan). HEPES-buffered saline (HBS), which contained 10 mmol l⁻¹ HEPES, 150 mmol l⁻¹ NaCl, 1 mmol l⁻¹ EDTA, and 0.005% Tween 20, pH 7.4 was used as eluent and diluting buffer for all experiments. Avidin and 5-[5-(*N*-succinimidyl-oxycarbonyl)pentylamido] hexyl-D-biotinamide [Biotin-(AC₅)₂-OSu] were purchased from Wako Pure Chemicals (Osaka, Japan). Bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Sigma-Aldrich (Tokyo, Japan).

Biotinylation of cultured cells and confocal laser scan micrograph

HeLa cells were obtained from Riken Cell Bank (Tsukuba, Japan). They were cultured in Eagle's

minimum essential medium (MEM) (Nissui Pharma., Tokyo, Japan) supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO₂ atmosphere. The cells were incubated in culture dishes (diameter: 10 cm) until reaching confluency. They were rinsed twice with ice cold phosphate-buffered saline (PBS), pH 7.4 after removing the medium. The cells scraped from 5 dishes were combined and suspended in HBS (pH 8.6) at a cell density of 5 × 10⁵ cells ml⁻¹. Fifty µl of biotin-labeling reagent [5 mg Biotin-(AC₅)₂-OSu in 1 ml dimethyl sulfoxide (DMSO)] was added to 1 ml of the suspended cells, and they were incubated for 30 min at 4 °C with mild shaking. The biotinylated cells were centrifuged at 1,000 × *g* for 2 min, and the pellet was rinsed three times with ice-cold PBS. The biotinylated cells were suspended into 1.49 ml HBS (pH 7.4) with 0.5% Tween 20. The cell suspension was mixed with 10 µl of Sigma proteinase inhibitor cocktail [bestatin, leupeptin, aprotinin, EDTA, 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), and trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), obtained from Sigma-Aldrich] followed by sonication for 15 s by Branson model 450D (Emerson Japan, Kanagawa, Japan), and was stored at -20 °C until use.

MOLT-4 cells were also obtained from Riken Cell Bank. They were cultured in RPMI1640 medium (Nissui Pharma.) supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO₂ atmosphere. They were biotinylated as described above for HeLa cells. They were fixed by 2% paraformaldehyde in PBS for 1 h at 25 °C and washed with PBS three times. And then they were incubated with PBS containing 1% BSA for 1 h at 37 °C and washed with PBS three times. And they were treated with PBS containing 0.05% Tween 20 for 5 min, and then stained with 500-fold diluted fluorescein-avidin D (Vector laboratories, Burlingame, CA) with PBS containing 1% BSA. The biotinylated and fluorescence-stained MOLT-4 cells were observed with a confocal laser scan microscope model Multiprobe 2001 (Molecular dynamics, Sunnyvale, CA).

Preparation of standard samples and samples for validation

The titer of the membrane sample (1 ml) prepared with 50 µl of biotin-labeling reagent as mentioned

above was defined as 50 unit ml⁻¹ of BP. The titer of the membrane sample prepared without the biotin-labeling reagent was defined as 0 unit ml⁻¹ of BP. The standard samples were prepared by mixing HBS (pH 7.4) and both 50 unit ml⁻¹ and 0 unit ml⁻¹ of BP, with an appropriate ratio (Table 1). Respective standard samples contained an identical amount of HeLa cell homogenates, but they contained various BP titers ranging from zero to 2.0 unit ml⁻¹.

Samples for validation were also prepared (Table 1). Each group of samples for validation consisted of 3 samples including an identical amount of BP but different concentrations derived from HeLa cell homogenates.

Preparation of the surface of a sensor chip

Avidin was immobilized to the surface of a sensor chip as described elsewhere (Johnsson et al. 1991). A continuous flow of HBS (pH 7.4) was maintained over the surface. The carboxyl groups on the CM5 sensor chip were activated with 0.2 mol l⁻¹ *N*-ethyl-*N'*-(3-diethylamino-propyl) carbodiimide

(EDC) and 0.05 mol l⁻¹ *N*-hydroxysuccinimide (NHS) for 9 min. Avidin was diluted to 100 µg ml⁻¹ in 10 mmol l⁻¹ acetate buffer (pH 4.0), and injected onto the activated surface for 9 min. Immobilization of avidin was terminated by incubating with 1 mol l⁻¹ ethanolamine-HCl for 9 min to block free ester groups. A flow-rate of HBS as a running buffer was 10 µl min⁻¹.

SPR experiments

One set of the SPR experiment consisted of 6 standard samples, and 9 samples for validation. Turn of injection of the standard samples and the samples for validation must be at random. The sample was injected into the apparatus for 1 min, and the washout solution (2 mol l⁻¹ guanidine-HCl plus 0.5% Triton X-100) was subsequently injected for 1 min to remove molecules bound non-specifically to the avidin-immobilized surface. Three minutes after the injection of the washout solution, the RU value was measured, followed by regeneration of the surface by 100 mmol l⁻¹ NaOH for 1 min. The net

Table 1. Preparation of standard samples and samples for validation.

Sample name	10 unit ml ⁻¹ of BP solution ^a (%)	0 unit ml ⁻¹ of BP solution ^b (%)	HBS Buffer (%)	Final BP conc. (unit ml ⁻¹)
Standard sample 1 ^c	0	20	80	0
Standard sample 2 ^c	2.5	17.5	80	0.25
Standard sample 3 ^c	5	15	80	0.5
Standard sample 4 ^c	10	10	80	1.0
Standard sample 5 ^c	15	5	80	1.5
Standard sample 6 ^c	20	0	80	2.0
Group 1 ^d				
Sample for validation 1	4	0	96	0.4
Sample for validation 2	4	12	84	0.4
Sample for validation 3	4	28	72	0.4
Group 2 ^d				
Sample for validation 4	8	0	92	0.8
Sample for validation 5	8	12	80	0.8
Sample for validation 6	8	28	64	0.8
Group 3 ^d				
Sample for validation 7	12	0	88	1.2
Sample for validation 8	12	12	76	1.2
Sample for validation 9	12	28	60	1.2

HBS (pH 7.4) containing 0.005% Tween20 and 1% EDTA was used as dilution buffer. Fifty unit ml⁻¹ of BP solution was prepared from HeLa cells biotinylated with 50 µl biotin-labeling reagent and 0 unit ml⁻¹ of BP solution was prepared as above without the biotin-labeling reagent. Respective standard samples contained an identical amount of homogenates from HeLa cells. (See Materials and methods).

^a 5-fold dilution of HeLa cell homogenates of 50 unit ml⁻¹ of BP; ^b 5-fold dilution of HeLa cell homogenates of 0 unit ml⁻¹ of BP; ^c The same amounts of non-specific components were contained in the respective standard samples; ^d In each group, samples for validation contained the same amounts of BP but various amounts of the non-specific components.

RU of each sample was calculated by subtracting the RU value obtained before sample injection from the RU value obtained after injecting the washout solution. All experiments were performed at 25 °C, at a flow-rate of 10 $\mu\text{l min}^{-1}$ of the running buffer (HBS).

Calibration of SPR data

The amounts of BP bound to the avidin-immobilized SPR-surface were gradually attenuated with repeated injection-regeneration cycles. To overcome this problem and to evaluate the exact amount of BP in the sample, a calibration rule was applied. One unit ml^{-1} of BP was injected at the end of each test to measure the ratio of attenuation, and the observed BP titer was read from the non-calibrated standard curve, the titer of which was expressed as *B*. Generally *B* is lower than 1 unit ml^{-1} . Assuming that each sample injection would equally attenuate the binding, the attenuation ratio (*A*) given by one sample-injection was defined as Equation (1).

$$A = (1 - B)/N_{\text{Total}} \quad (1)$$

$$(\text{Calibrated RU value}) = C/(1 - A \cdot N_{\text{Turn}}) \quad (2)$$

where N_{Total} is the total number of the repeated sample injections on an avidin-immobilized chip; N_{Turn} is an injection turn number of a sample, the titer of which should be calibrated; and *C* represents the measured RU value read for the sample read from the standard curve without calibration. Accordingly, an RU value observed was calibrated by using Equation (2) to correct the effect of the attenuation. The calibrated standard curve was created from the calibrated RU values obtained with the standard samples, and finally the BP concentration was calculated from the calibrated standard curve.

Purification of plasma membrane of HeLa cells and others

Plasma membrane of HeLa cells were purified by the method of Nesbitt and Horton (1992) with some modifies. HeLa cells were placed into 10 ml of cold, homogenizing buffer (25 mmol l^{-1} Tris, 250 mmol l^{-1} sucrose, 10 μl of Sigma proteinase

inhibitor cocktail, pH 7.4). Cells were homogenized with 30 strokes by the Potter–Elvehjem homogenizer. Homogenized suspension was centrifuged at $10,000 \times g$ for 30 min at 4 °C to remove debris. The supernatant was separated, followed by adding 100 mmol l^{-1} NaCl and 0.2 mmol l^{-1} MgSO_4 , and was incubated for 30 min at 4 °C. The solution was centrifuged at $15,000 \times g$ for 5 min at 4 °C, and then the supernatant was centrifuged at $50,000 \times g$ for 30 min at 4 °C. The precipitate was the plasma membrane fraction.

Aminopeptidase N activity was assayed in 150 mmol l^{-1} 3,3-dimethylglutaric acid (GTA) buffer (pH 8.0) with L-leucine-*p*-nitroanilide as the substrate (Olsen et al. 1988; Look et al. 1989). Protein concentrations were determined by the Bradford method (1976), using BSA as the standard.

Results and discussion

Distribution of biotinylated proteins of MOLT-4 cells

Figure 1 shows micrographs of the biotinylated and fluorescence stained MOLT-4 cells by a confocal laser scan microscope. MOLT-4 cells without biotinylation showed no fluorescence (Figure 1d). On the other hand, the biotinylated MOLT-4 cells had fluorescence only on the peripheral areas of the cells (Figure 1e). This demonstrates that the proteins only on the plasma membrane were biotinylated. In contrast, the MOLT-4 cells treated with 0.05% Tween 20 before biotinylation showed fluorescence even inside of the cells (Figure 1f). It is considered that membrane pores might be formed by the treatment with 0.05% Tween 20 and that cytoplasmic proteins were biotinylated. The method proposed here for biotinylation of the cells might biotinylate only membrane proteins, and thus the BP titer could be an index for the membrane purification.

SPR experiments

Figure 2 shows typical sensorgrams observed in the present procedures. A sample solution was injected to an avidin-immobilized surface. The washout solution was subsequently injected to

Figure 1. Micrographs of biotinylated and fluorescence stained MOLT-4 cells by a confocal laser scan microscope. Cells were fixed and stained by fluorescein avidin D. (a), (b), and (c) are scanings for transmitted light and (d), (e), and (f) are scanings for fluorescence (EX: 488 nm, EM: 510 nm). (a) and (d) MOLT-4 cells without biotinylation. (b) and (e) Biotinylated MOLT-4 cells. (c) and (f) Biotinylated MOLT-4 cells with a 0.05% Tween 20 treatment before biotinylation. Bar represents 10 μm .

remove molecules bound non-specifically to the surface. Three minutes after the injection of the washout solution, the RU value was measured, and then the surface was regenerated by injecting 100 mmol l^{-1} NaOH for 1 min. The RU value for the sample was evaluated by subtracting the RU value observed before the sample injection from that after the injection of washout solution. Although the regeneration procedure described above seemed to be incomplete and insufficient, the harsher treatment has not been considered because of the lifetime of the avidin-immobilized surface.

At first, we had used a reference surface on which BSA was immobilized, and tried to determine the concentration of BP from data by subtracting RU value on a BSA-immobilized surface from that on the avidin-immobilized surface. This reference system worked well when the number of the sample injection was smaller, but the degrees of the non-specific binding of the membrane components to the surfaces deviated considerably in repeated experiments with increase in the number of injection (Data not shown). Therefore, we designed a method for determining BP titer as described above in which the reference surface was

not needed. Purified BSA and OVA were biotinylated and injected on to the avidin-immobilized surface and followed by the injection of washout solution, and it was found that the RU values were identical before and after the injection of washout solution (Data not shown). Accordingly, the avidin-biotin binding was confirmed not to be dissociated by the washout treatment.

Calibration of SPR data

Figure 3 shows a typical standard curves before and after calibration. Figure 3a shows that the RU values obtained from injections in early turns were larger than the regression line (e.g., the injection of the standard BP sample of 0.5 unit ml^{-1} was done at the 4th turn, and that of the 1.5 unit ml^{-1} was at the 7th turn), and the RU values obtained in the latter turns were smaller than the regression line (e.g., the injection of the standard BP sample of 0.25 unit ml^{-1} was done at the 14th turn, and that of the 2.0 unit ml^{-1} was at the 15th turn). A correlated coefficient of the standard curve before calibration was 0.937, and it was improved to 0.998 after calibration.

Figure 2. Five sensorgrams obtained from five standard samples (0, 0.25, 0.5, 1, and 2 unit ml^{-1} of BP) are represented. At first, sample was injected for 1 min, and then the washout solution (2 mol l^{-1} guanidine hydrochloride plus 0.5% Triton X-100) was subsequently injected for 1 min to remove the molecules non-specifically bound to the avidin-immobilized surface. Then, the surface was regenerated by 100 mmol l^{-1} NaOH for 1 min. All sensorgrams are adjusted to zero at the point of just before the sample injection. Therefore, the RU value at the measurement point, namely, 3 min after the injection of the washout solution, is considered to represent the amount of BP specifically bound to the surface. The flow-rate of HBS as a running buffer was $10 \mu\text{l min}^{-1}$. All experiments were performed at 25°C . Open and closed arrows show, respectively, the start and the end of the injections of the respective solutions.

This calibration protocol is based on the idea that the attenuation was generated with arithmetic series (see *Calibration of SPR data* of Materials and

methods). Many other ideas could be adopted, for example, the attenuation was generated with geometric series or inhibition by remaining biotinylated proteins. However, the simple calibration system based on the idea of arithmetic series was considered adequately applicable to our experiments.

Validity of determination

In order to check the accuracy of determination, ratios of the calculated value to the theoretically-estimated value of the BP titer were examined using the membrane samples prepared for validation. In this case, when the ratio is 1, the calculated value of the BP titer is the same as the theoretically-estimated one. Figure 4 shows averages of the ratios for 9 samples for validation before and after the calibration through 4 sets of determination successively carried out on an avidin-immobilized surface. Bars show the levels of standard deviation (SD). Until the third set of the determination, both ratio and SD were improved by the calibration from 0.92–1.04 (SD: 0.09–0.23) to 1.00–1.01 (SD: 0.04–0.09). The values obtained after calibration indicates that the calculated BP titer and the theoretically-estimated one are substantially equal, suggesting that the BP data obtained by the calibration method could be accurate at least until the third set of the determination. In the fourth set, the calibration also improved both ratio and SD, whereas the ratio

Figure 3. Typical standard curves before (a) and after (b) calibration. The RU values of initial part of injections were larger than the regression line (e.g., the standard sample of 0.5 unit ml^{-1} of BP was at the 4th injection, and that of 1.5 unit ml^{-1} of BP was at 7th injection) and RU values of the posterior part were smaller (e.g., 0.25 unit ml^{-1} of BP at the 14th injection, and 2.0 unit ml^{-1} of BP at the 15th injection). A correlated coefficient of the standard curve before calibration was 0.937. And it was improved to 0.998 after calibration.

Figure 4. Stability of an avidin-immobilized surface through successive 4 sets of determination on a surface. Ratios of the calculated value of the BP concentration to the theoretically-estimated value of the BP concentration of the validation samples were examined to test a stability of the avidin-immobilized surface. The ratio 1 means that the calculated value and the theoretically-estimated one are the same. Results are expressed as the mean $\pm$ SD for 9 samples for validation before (shaded bars) and after (solid bars) calibration.

and SD were >1.5 and >0.5 , respectively after the calibration, suggesting that the BP determination in the fourth set was unavailable.

Figure 5 shows the standard curves of the successive 4 sets of determination without calibration. A slope of the standard curve in the fourth set was attenuated to one eighth of that in the first set, and a correlation coefficient of the standard curve of the fourth set was 0.91 even after calibration, being far less than 1.00, and might be too small to be corrected by the calibration method. This insufficient small value may be caused by the decrease in amounts of biotinylated protein bound to the avidin-immobilized surface.

The avidin-immobilized surface used in the present study was regenerated by 100 mmol l^{-1} NaOH, although the regeneration was incomplete and sensorgrams were not restored to the base line (Figure 2), suggesting that there remained some biotinylated proteins bound to the surface even after the regeneration procedure. This might account for decreasing the binding of biotinylated proteins to the sensor surface. The incomplete regeneration would be a disturbing factor for determining BP, while the method adopted has been conducted well as a whole.

To improve the regeneration, other regeneration solutions were examined, for example, 8 mol l^{-1} Urea, 100 mmol l^{-1} HCl, 10% DMSO, 100 mmol l^{-1} glycine-HCl (pH 2.5), 1% SDS, and 100 mmol l^{-1} phosphoric acid, but the attenuation in the BP binding in the repeated injection onto the avidin-immobilized surface could not be improved in comparison with that by 100 mmol l^{-1} NaOH (Data not shown). Treatment of the surface with more drastic conditions could be possible, but this might cause denaturing the immobilized avidin.

The surface lifetime is desired to be more improved because of a high cost of the sensor chips. To resolve this issue, resetting an avidin-immobilized surface by re-immobilization with avidin may be effective for recycling the chip.

Evaluation of BP concentration as an index during membrane purification

The BP titer determined was compared with aminopeptidase N activity as an index of membrane purification of HeLa cells. Aminopeptidase N is a metal peptidase on plasma membrane and it is also identical to human surface antigen CD13, which

Figure 5. Standard curves of the successive 4 sets of determination without calibration on an avidin-immobilized surface. The standard samples were prepared by mixing HBS, 50 unit ml⁻¹ and 0 unit ml⁻¹ of BP, with an appropriate ratio (Table 1). Square: First set of determination, Diamond: Second set of determination, Triangle: Third set of determination, Circle: Fourth set of determination.

has been used so far for characterizing and typing leukemia or lymphoma cells (Olsen et al. 1988; Look et al. 1989). Figure 6 shows the ratios of aminopeptidase N activity and BP titer of each fraction to those in various cell homogenates. Each purification ratio of fractions by the BP titer was almost equal to that by the aminopeptidase N activity and there were no significant difference ($p > 0.01$) between BP titer and aminopeptidase N activity by unpaired student's *t*-test.

Figure 6. BP titer was compared with aminopeptidase N activity as an index during membrane purification process of HeLa cells. Purification ratio shows the ratios of aminopeptidase N activity and BP concentration of each fraction to that of whole cell homogenates.

Conclusions

The results clearly show that the combination of the whole cell biotinylation and the BP titer measured by SPR biosensor could be used as an index of membrane purification. It was visually identified that the biotinylated proteins were existed only in the peripheral areas of the cells. Cells are often biotinylated to identify cell surface molecules instead of radioactive isotopes, and followed by immunoprecipitation or western blotting, and now they could be also applied for membrane purification as an index. Furthermore, it would be applicable for most kind of cells without considering about expression or distribution of enzymes on the cells or inactivation of marker enzymes during purification.

By the concise calibration, the total amounts of biotinylated proteins were quantified by SPR biosensor with high accuracy. The methods requires no reference surfaces on the sensor chip, thus, it is able to determine using half volume of samples for half time. Although the surface lifetime was not so long, recycling of the sensor chip by re-immobilization with avidin could be expected for reductions in cost.

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