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A simple method to prepare a biopterin sensor chip applicable to crude samples

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Biopterin (BP), which is structurally similar to flavin, is potentially associated with a UV-B photoreceptor yet to be identified. The stagnation of research on the BP-associated photoreceptor is because of the lack of a simple method to detect the protein that interacts with BP. We successfully immobilized BP by conducting a coupling reaction between one of the activated hydroxyl groups of BP and pre-introduced primary amino groups on the CM5 surface at a pH higher than usual. This enabled us to detect the protein that interacts with BP using Biacore measurements. The validity of the BP sensor chip was tested by applying the chip to fractions obtained by subjecting a crude extract of the paraflagellar body (PFB), which is the photosensory organelle of *Euglena*, to an anion exchange column; this test was performed because PFB was spectroscopically shown to contain a pterin binding protein. The BP sensor chip was useful to detect an unknown BP-binding protein in a crude sample.

Keywords: Biacore, Biopterin-binding protein, Biopterin-sensor chip, paraflagellar body (PFB), Pterin-type- UV-B photoreceptor, Tetrahydrobiopterin

Introduction

Since the 1980s, the increase in UV-B (280–315 nm) radiation reaching the Earth's surface because of the stratospheric ozone depletion has been considered to be a serious problem for life. Plants have developed various survival

strategies against this environmental stress by stimulating protection and repair mechanisms. Terrestrial plants accumulate various UV-absorbing substances, such as flavonoids, anthocyanins, hydroxycinnamic acid esters, and sinapic acid esters, in epidermal cell vacuoles in response to the UV-B radiation (Burchard et al. 2000; Chapple et al. 1992; Schwinn et al. 2009). All of these substances are products of the phenylpropanoid pathway, its branching

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pathway, and the flavonoid pathway. First, phenylalanine ammonia lyase (PAL), a key enzyme in these pathways, was introduced into a terrestrial plant ancestor by horizontal gene transfer directly from a bacterium or possibly via the Dikarya fungi (Emiliani et al. 2009). Subsequently, terrestrial plants developed several secondary metabolic pathways to adapt themselves to various terrestrial ecosystems. Among them, the light-induced anthocyanin accumulation has been extensively studied in various plants, such as grape, strawberry, and maize. The action spectra for anthocyanin accumulation indicate the presence of a specific UV-B photoreceptor with a chromophore that may be a reduced pterin compound (Galland and Senger 1988; Takeda 1988; Yatsushashi et al. 1982).

To elucidate the molecular identity of the putative UV-B photoreceptor, we determined the action spectra of anthocyanin accumulation and PAL and chalcone synthase gene expressions in cultured carrot cells (Takeda and Abe 1992; Takeda et al. 1994), and we examined the effects of a pterin biosynthesis inhibitor on these UV-B responses (Takeda et al. 2014). The results indicated that the putative UV-B photoreceptor has an unconjugated pterin, tetrahydrobiopterin (H₄BP), as its chromophore.

In 2002, the UV-resistant locus 8 (*UVR8*) in *Arabidopsis* was cloned (Kliebenstein et al. 2002), and its product was found to be a novel UV-B photoreceptor (Christie et al. 2012; Rizzini et al. 2011; Wu et al. 2012). Because the chromophores W285 and W233 of *UVR8* were elucidated, we

re-examined the action spectra of the anthocyanin synthesis, the *PAL* and *HYS* transcripts (Brown et al. 2009). We demonstrated that all action spectra were accounted for by the sum of the *UVR8* (280 nm peak) and H₄BP (ca. 300 nm peak) absorption spectra. Thus, we concluded that the anthocyanin synthesis is co-regulated by *UVR8* and H₄BP-type photoreceptors (Takeda et al. 2014).

For the biochemical identification of the H₄BP-type photoreceptor, the biopterin (BP)-binding activities of candidate proteins should be measured in a simple and rapid manner. We used a real-time interaction analysis system such as Biacore (GE Healthcare) for this purpose. Because the preparation of BP-sensor chip using the manufacturer's directions was unsuccessful (Takeda et al. 2013), we tried to introduce a primary amino group to one of the hydroxyl groups at the BP propyl side chain that was applicable to the conventional amine coupling method (Takeda et al. 2013). However, the new method was too complex for practical use.

In the present study, we report an effective and simple method to construct a Biacore sensor chip that senses BP (BP sensor chip). The validity of the BP sensor chip was tested by applying it to the paraflagellar body (PFB)-rich fraction of *Euglena* because the presence of pterin-binding protein in PFB has been spectroscopically demonstrated (Häder and Lebert 1998).

Materials and methods

Culture conditions

Euglena gracilis strain Z (NIES-48) was cultured in a heterotrophic growth medium [0.14 mM KH₂PO₄, 0.1 mM MgSO₄·7H₂O, 4.8 mM sodium acetate, 0.13 mM potassium citrate, 0.6 g l⁻¹ bactopeptone (Difco, Detroit, MI, USA), 0.4 g l⁻¹ yeast extract (Difco), 1.3 μM vitamin B₁, and 370 pM vitamin B₁₂] under continuous white light illumination using a 20 W white fluorescent tube at 27°C.

Isolation of the PFB-rich fraction and protein extraction

Euglena cells cultured for 7 days (5×10^5 cells ml⁻¹) were harvested from a 3-l culture and washed once with 50 ml of 50 mM potassium phosphate buffer containing 0.5 mM EDTA (pH 7.0). The cells were resuspended in the same buffer with 0.3 M sucrose. A 0.3-ml aliquot of the suspension was placed in a 1.5-ml microtube and disrupted three times with the Biorupter UCD-200TM (Tosho Denki Co. Ltd.) at a low setting at 0.5- and 1.5-min intervals. Then, 70% of the cells were disrupted. After vigorous shaking and centrifugation at $400 \times g$ for 15 min at 4°C, the undisrupted cells and large cell debris were removed. The small particles, including PFBs and fragments of flagella, were then precipitated by centrifugation at $6,500 \times g$ for 15 min at 4°C and stored at -80°C.

The precipitated pellets containing PFB and fragments of flagella were resuspended in 0.3-ml of 40 mM Tris-HCl lysis buffer at pH 8.0, containing 0.2% *n*-dodecyl-β-D-maltoside,

10 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and disrupted with the same Biorupter but at a high setting. After centrifuging the lysate at $10,000 \times g$ for 10 min at 4°C, the supernatant was used as the crude extract. The crude extract was desalted on a PD-10 desalting column (Amersham Biosciences, Uppsala, Sweden).

Fractionation of the crude extract on an anion exchange column

The crude extract was applied to an anion exchange column (Toyopearl CM-650M; Tosoh Co. Ltd) equilibrated with a 0.1 M Tris-HCl start buffer, pH 8.0. After washing with the start buffer, 50 fractions of 1 ml each were eluted using a linear NaCl gradient (0–0.5 M) containing 0.1 M Tris-HCl, pH 8.0.

Preparation of the activated BP

The ligand (BP) was activated in dimethyl sulfoxide (DMSO) by incubation with disuccinimidyl carbonate (DSC) and *N,N'*-dimethylaminopyridine (DMAP) for more than 2.5 days. The molar ratio of BP:DSC:DMAP was 1:4:1, as previously described (Takeda *et al.* 2013). The reaction scheme is shown in Fig. 1. The activated BP [succinimidyl-carbonyl-BP, BP-O-C(=O)-OSu] product remained stable in DMSO under dark conditions for more than 3 months.

The reagents used above, BP, DSC, DMAP, and dried DMSO (Wako Pure Chemical Industries Ltd., Osaka, Japan), and their stock solutions were stored in a desiccator before use

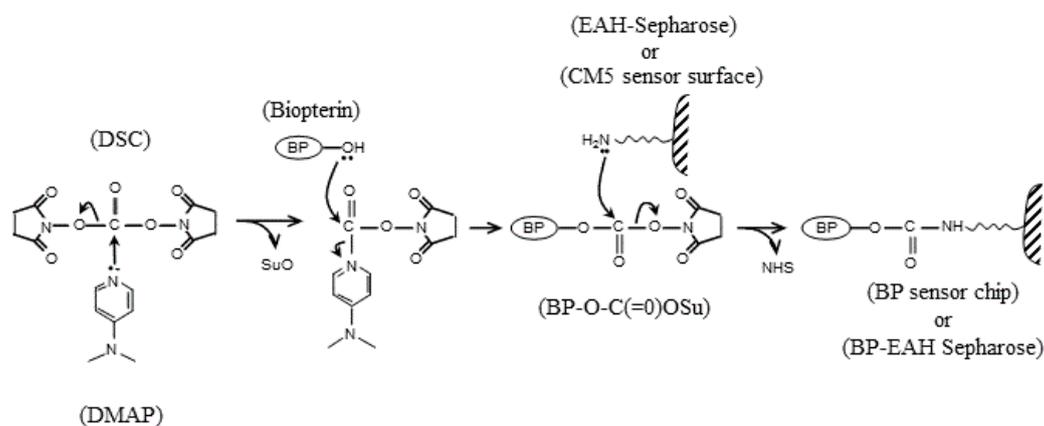


Fig. 1 Reaction scheme for preparing the biopterin (BP)-sensor chip or BP-EAH Sepharose gel

Some of the carboxyl groups on the test flow cell surface of the CM5 sensor chip were changed to primary amino groups. NHS, *N*-hydroxysuccinimide or SuOH; DSC, *N,N'*-disuccinimidyl carbonate or SuO-C(=O)-OSu; DMAP, dimethylaminopyridine.

Preparation of BP-EAH Sepharose gel

EAH-Sepharose 4B (7–12 $\mu\text{mol NH}_2/\text{ml}$ gel; GE Healthcare Bioscience AB, Uppsala, Sweden) was washed with MilliQ water (EMD Millipore, Billerica, MA, USA) and equilibrated with 100 mM Na_2CO_3 . An equal volume (2 ml) of activated BP and EAH-Sepharose solution (1 g/2 ml) was mixed and manually agitated for 30 min to prepare the BP-EAH Sepharose gel. The mixed solution was then adjusted to 4 ml with distilled water, and the gel was collected by centrifugation and repeatedly washed with 100 mM Tris buffer, pH 8.

Preparation of the BP-sensor chip

The BP-sensor chip was made using a CM5 sensor modified to carry free amino groups by

amine coupling according to the manufacturer's instructions. Activated BP was mixed with buffer (100 mM Na_2CO_3 , pH 10) at a 1:1 ratio and quickly injected over the amine surface to prevent hydrolysis, and it was allowed to react for 30 min.

Biacore measurements

The Biacore measurements were taken using the Biacore X system equipped with the CM5 sensor chip (research grade), HBS buffer (10 mM Hepes with 0.15 M NaCl, 3.4 mM EDTA, and 0.005% P20 surfactant, pH 7.4), and an amine coupling kit provided by the manufacturer (GE Healthcare Bio-Sciences KK, Tokyo, Japan). The other conditions were set according to the manufacturer's instructions.

The results of the measurements are shown in response units (RU) as a function of time on a

sensorgram. The RU values were obtained by subtracting the RU1 value of the reference flow from the RU2 value of the BP-immobilized test flow. The amount of proteins bound to the BP was obtained from the sensorgram because 1000 Δ RU corresponded to 1 ng protein.

Hydrophilic interaction liquid chromatography

The BP and reaction intermediate, BP-O-C(-O)-OSu, were fractionated using hydrophilic interaction liquid chromatography (Shiseido Co. Ltd., Tokyo, Japan) under the following conditions: column size and eluents were 4.6 mm id \times 250 mm and 10 mM HCOONH₄ in 70% CH₃CN, respectively. The flow rate, temperature, and absorption wavelength were 1.0 ml min⁻¹, 28°C, and 360 nm, respectively. The elution times for BP and BP-O-C(=O)-OSu under these conditions were 7–8 min and 4–5 min, respectively.

Treating the samples with BP-EAH Sepharose or EAH-Sepharose gel and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE)

A 0.5-ml aliquot of each fractionated sample was mixed with 0.5-ml of BP-EAH Sepharose or EAH-Sepharose gel equilibrated with 100 mM Tris HCl (pH 8) and agitated for 24 h with a Mini Disk Rotor (Bio Craft Co. Ltd., Tokyo, Japan) in a cold room at 4°C to prevent the precipitation of the gel. The gel was collected

by centrifugation at 9,000 rpm for 1 min, and the supernatant was analyzed by SDS-PAGE. The gels were then stained using a silver-staining kit (GE-Healthcare Bioscience). The protein concentration was determined using the Bradford (Bio Rad Laboratories Inc.) or micro BCA reagent (Thermo Fisher Scientific Inc.).

Results

We previously reported that succinimidyl-carbonyl-BP [BP-O-C(=O)-OSu], an activated hydroxyl group of BP, effectively couples to a primary amino group of a diamine at high pH (Takeda *et al.* 2013). In the present study, we applied the same coupling reaction between BP-O-C(=O)-OSu and amino groups on a CM5 sensor chip to prepare the BP-sensor chip.

The reaction scheme for preparing the BP-sensor chip is shown in Fig. 1. The reaction begins with a nucleophilic attack from the nitrogen of the DMAP pyridine ring to the DSC carbonyl. Then, an unshared oxygen electron pair in the BP hydroxyl group attacks the carbonyl carbon, forming the activated compound BP-O-C(=O)-OSu; these reactions occur in DMSO. Finally, the amino residue in the test flow cell couples with the activated compound in 50% DMSO (pH 10), creating the BP-sensor chip.

As it was difficult to determine how much BP was introduced to the sensor surface, we applied the same coupling reaction between BP-O-C(=O)-OSu and EAH Sepharose at pH 10 for 30 min. The reaction products were analyzed by high-performance liquid chromatography (HPLC) (Fig. 2). BP-O-C(=O)-OSu is shown as a peak, eluting at about 4 min (Fig. 2b). The peak was fork shaped, indicating that the product contained two structural isomers, probably derived from the two hydroxyl groups in BP. BP-O-C(=O)-OSu either rapidly reacted with the EAH Sepharose primary amino groups or was hydrolyzed to BP. Thus, only the BP peak was observed in the supernatant after centrifugation (Fig. 2c). The amount of BP incorporated into the gel was calculated from the difference in the BP peak area before (a) and that after (c) the coupling reaction. The

BP-EAH Sepharose gel thus obtained had 1.8 μmol BP/g gel, which was about 20% of the total number of amino groups on the EAH-Sepharose gel. A comparable amount of BP bound to EAH Sepharose was expected to bind to the amino groups on the surface of the CM5 sensor chip. Here the number of primary amino groups immobilized onto the test flow cell was 0.33–1.75 pmol (1.06 ± 0.09 , mean \pm standard deviation; $n = 4$), which was approximately half the amount of *N*-hydroxysuccinimide (NHS) molecules bound to the sensor surface using conventional activation of carboxyl groups on the CM5 sensor chip (2.68 ± 0.03 pmol; $n = 4$); thus, 0.21 pmol BP (range, 0.06–0.35 pmol) was immobilized on the chip surface.

Fig. 3 depicts a representative sensorgram for the crude extract from the PFB-rich subcellular fractions of *Euglena* using the BP-sensor chip.

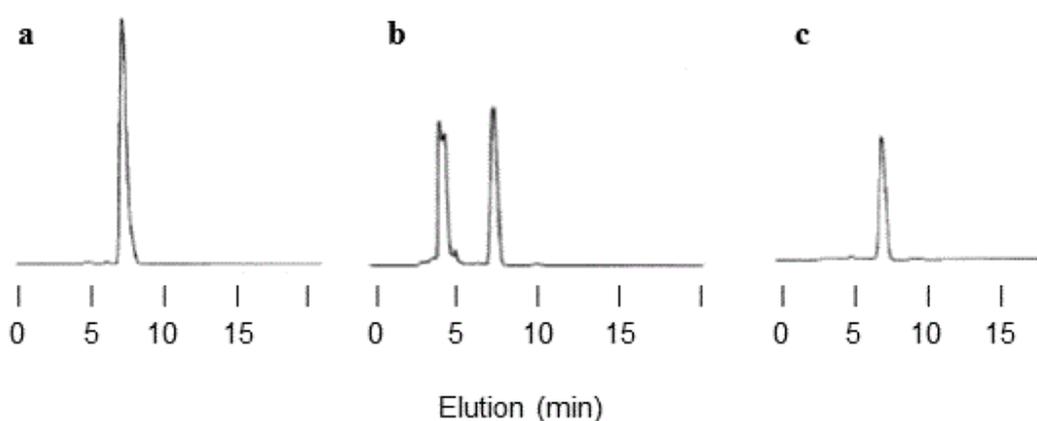


Fig. 2 Quantitative incorporation of bipterin (BP) into EAH-Sepharose gel

Hydrophilic interaction liquid chromatography profiles (eluted with 80% CH_3CN) for 12 mM BP (a), 12 mM BP activated with DSC in DMSO/DMAP (b), and the supernatant of the reaction mixture 30 min after mixing activated BP with EAH-Sepharose gel (c). Retention time and peak area (integrated value) are 7.577 min, 221420 (a); 4.255 min, 126558 and 7.608 min, 107979 (b); 7.444 min, 103401 (c), respectively.

When the sample solution was introduced into the flow at 75 s, an initial abrupt increase in the RU value occurred because of the bulk effect and was followed by a slow increase in the RU value, indicating BP-specific binding. The Flow was changed to the original buffer at 200 sec, and the RU value decreased with time to the original level after a large decrease (bulk effect). Here Δ RU reflected the amount of protein bound to the BP-sensor immediately before changing the flow. The Δ RU value proportionally decreased with the degree of the sample dilution to 1/2, 1/3 and so on. This observation indicates that the sensor chip can be applied to quantify the BP-binding protein.

The Δ RU value in Fig. 3 was 122, which

corresponded to 0.122 ng of protein. If we assume that the relative molecular mass of the protein was 5×10^4 , then 0.0024 pmol of the protein bound and occupied only 1% of the BP ligands (calculated from $-\text{NH}_2$ groups incorporated on the CM5 sensor). The BP-sensor chip could be used repeatedly with good reproducibility, even with a crude extract. Next, we applied the BP-sensor chip to the crude extract fractionated by anion exchange chromatography with a salt gradient (Fig. 4) and determined the Δ RU value for each fraction. The active fractions with high Δ RU values (peak at #30) eluted with NaCl at the concentration between 0.18 M (fraction #28) and 0.24 M (fraction #32).

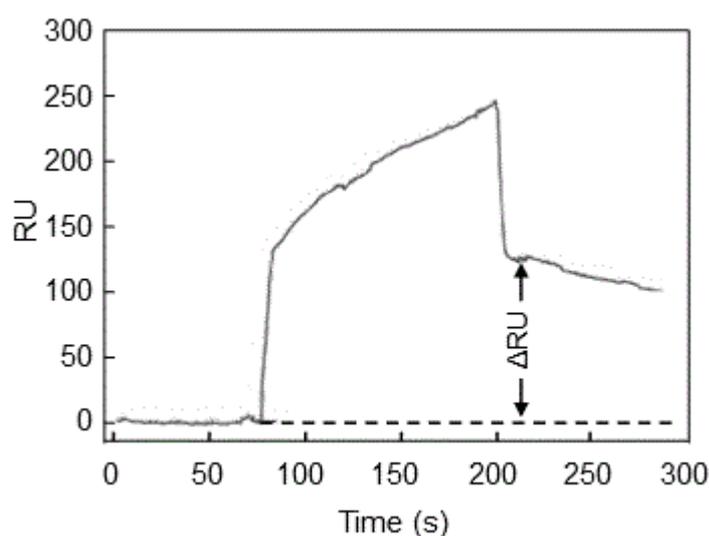


Fig. 3 Biacore sensorgram for the crude extract from paraflagellar body (PFB)-rich fractions of *Euglena*

The crude extract was treated with EAH-Sepharose gel overnight before measurements. The protein concentration was 0.48 mg/ml. Arrows (Δ RU) indicate specific interactions between proteins in the crude extract and the biopterin (BP) sensor chip.

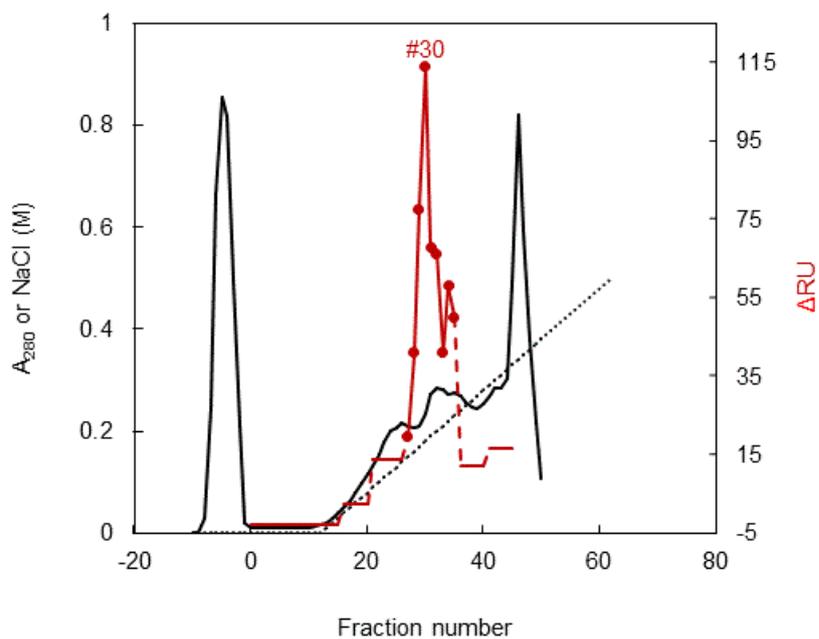


Fig. 4 Application of the biopterin (BP)-sensor chip to purify the crude extract from paraflagellar body (PFB)-rich fractions of *Euglena*

The eluent was monitored at an absorption wavelength of 280 nm (black), and the Δ response unit (RU) Biacore measurement was obtained (red). Dotted line indicates NaCl concentration.

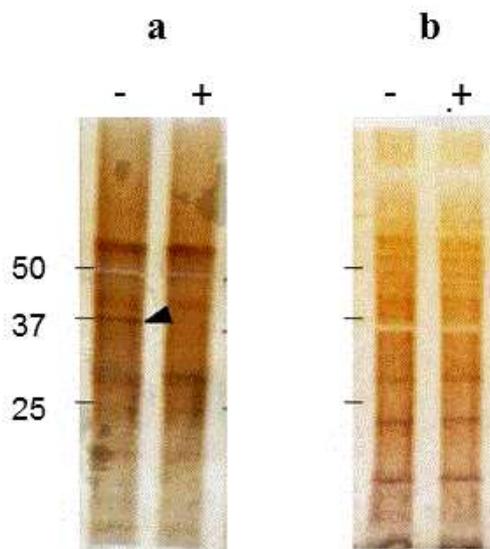


Fig. 5 The active fraction contained a 37 kD biopterin (BP)-binding protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

The active (28–32) (a) and inactive (20 and 21) (b) fractions shown in Fig. 4 were run on a 12% polyacrylamide gel. Each fraction was collected and treated overnight with BP-EAH Sepharose (+) or control EAH-Sepharose (–) gel before the run.

We examined whether or not these active fractions (#28–32) contained the BP-binding protein using BP-EAH Sepharose gel chromatography. As shown in Fig. 5a, the 37-kDa SDS-PAGE band of the active fraction disappeared after BP-EAH Sepharose gel (+) treatment, indicating that the 37-kDa band was a candidate BP binding protein in PFB. These results show the usefulness of the BP-sensor chip for simply and rapidly detecting unknown BP-binding proteins.

Discussion

According to the manufacturer's instructions of Biacore, activated compounds, both $-C-C(=O)-OSu$ (conventional amine coupling) and $-O-C(=O)-OSu$ (immobilization using $-OH$ groups), can be easily coupled to the $-NH_2$ group under mild conditions (pH 7.4 for the former and pH 7 for the latter). However, our attempts to prepare a BP-sensor chip according to the manufacturer's instructions were unsuccessful (Takeda *et al.* 2013). As shown previously, the reaction between the activated BP and $BP-O-C(=O)-OSu$ did not couple to the $-NH_2$ group within 30 min under mild conditions (pH 7 or 8), but it did couple at a higher pH (pH 10) (Takeda *et al.* 2013).

We noticed a crucial difference between $-C-C(=O)-OSu$ and $-O-C(=O)-OSu$ atoms that bound to the carbonyl carbon, i.e., C and O for the former and two O's for the latter. The oxygen next to the carbonyl-carbon atom is inductively electron-withdrawing, whereas it is electron-donating through resonance because of its lone pair of electrons. Because the

electron-donating effect of carbonyl-carbon atom through resonance is larger than the inductive electron-withdrawing effect, the electron density of it in $-O-C(=O)-OSu$ should be higher than that in $-C-C(=O)-OSu$. Thus, a higher pH is necessary for the $-NH_2$ group nucleophilic reaction with $-O-C(=O)-OSu$ when compared with that of $-C-C(=O)-OSu$. This seemed to be the main reason why we were unsuccessful in making the BP sensor chip according to the manufacturer's instructions.

The BP-sensor chip was constructed according to Fig. 1 on the basis of the previous report (Takeda *et al.* 2013). In the reaction of the activated BP with EAH Sepharose, the amount of BP bound was determined by the HPLC analysis as shown in Fig 2. According to that data, we estimated the amount of BP on the surface of the BP-sensor chip, which was sufficient for the repeated use of the sensor chip. Although our BP-sensor chip is not suitable to evaluate the kinetic parameters of the interactions between BP and proteins, the chip was sufficient to detect an unknown BP-binding protein in the crude extract. We detected a 37-kDa protein in the crude extract of the PFB-rich subcellular fraction using the BP-sensor chip.

Brodhun & Haeder (1995) reported about isolating pterin-binding proteins from PFB-flagella preparations with apparent molecular masses of 24,000–28,800 using liquid chromatography. However, the molecular identity of these proteins was not elucidated. In the present study, we could not detect these proteins probably because of the differences in

the preparation of the starting materials. Molecular identification of the 37-kDa protein that we detected will be the subject of a future study.

Acknowledgments

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