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Suzuki coupling for protein modification

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Abstract—Suzuki-coupling, a representative cross-coupling reaction between small molecules, can proceed on a protein surface in aqueous solution under mild conditions. The modified protein produced by the coupling reaction maintained its native like structure and function. In addition, fluorescent dye appended-protein acts as a fluorescent biosensor.
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Post-translational modification on protein surfaces is one of the major strategies for enrichment of protein structure and function in nature. Although such a strategy should be valuable in the research field of protein engineering, successful examples have been limited.[1] This is mainly due to that we still have only a few chemistry-based methods available to efficient chemical modification reactions toward proteins in spite of the well-developed synthetic organic chemistry. As a classical example, ketone–hydrazine chemistry[2] has been widely used for attachment of nonnatural molecules to native proteins. The validity of azide-based chemistry such as Staudinger reaction[3] and Huisgen reaction[4] was recently discovered as an orthogonal reaction by several groups and now applied to many fields. Diazonium coupling and Mannich reaction have been also developed as modification methods of tyrosine residues on protein surfaces.[5] With the aim of multiple-modification and the sophisticated functionalization of proteins, various chemistry orthogonal to each other are greatly desirable. Cross-coupling reaction is one of the representative synthetic methods in the modern organic chemistry.[6] The pioneered examples on utilization of the cross-coupling (Sonogashira reaction) toward peptides in aqueous solution were reported by Schmidtchen and Ghadiri.[7] However, the functionalization of the resultant peptides after coupling reaction was not shown clearly. Although a few examples of the Suzuki-coupling reaction, a typical Pd-catalyzed cross-coupling reaction, toward short peptides were reported recently, these reactions were performed only in organic solvents.[8] Here we describe that Suzuki-coupling can proceed under mild aqueous conditions to give a modified protein with retaining its natural structure and function. Furthermore, it is demonstrated that a fluorophore-appended WW domain protein thus prepared, acts as a fluorescent biosensor for peptides bearing phosphoserine.

In this study, the WW domain portion of Pin1 protein (residues 6–39) was chosen as a model protein of Suzuki-coupling reaction on protein surface. The WW domain is a protein module mediating protein-protein interaction in a cell signal transduction cascade.[9] As a typical Suzuki-coupling reaction in aqueous solution, 4-iodophenylalanine-appended WW domains (WW iFs) and boronic acid derivatives (**1**–**4**) were employed as a protein substrate and coupling reagents, respectively (See Scheme 1). Both native WW domain and WW iFs were synthesized by solid phase peptide synthesis and identified by MALDI-TOFMS.

As an initial attempt, 3,5-bis(trifluoromethyl)phenylboronic acid (**1**), a highly reactive substrate, was used to optimize reaction conditions in the presence of water soluble palladium salt (Na_2PdCl_4 as a catalyst) without any phosphine ligands. The coupling reaction of WW iF34 with **1** in 10 mM Tris–HCl buffer solution (pH 8.0) was monitored by reversed phase HPLC (Fig. 1a). A new peak eluted after the peak of WW iF34 (14.5 min) appeared at 16.6 min that was identified by MALDI-TOFMS (Fig. 1b) as a coupling product, WW F34-**1** (*m*/*z* 4383.5 $[M+H]^+$, calcd 4384.4, together with the dication form, *m*/*z* 2192.3 $[M+2H]^{2+}$, calcd 2192.7). Since the native WW domain did not give the coupling

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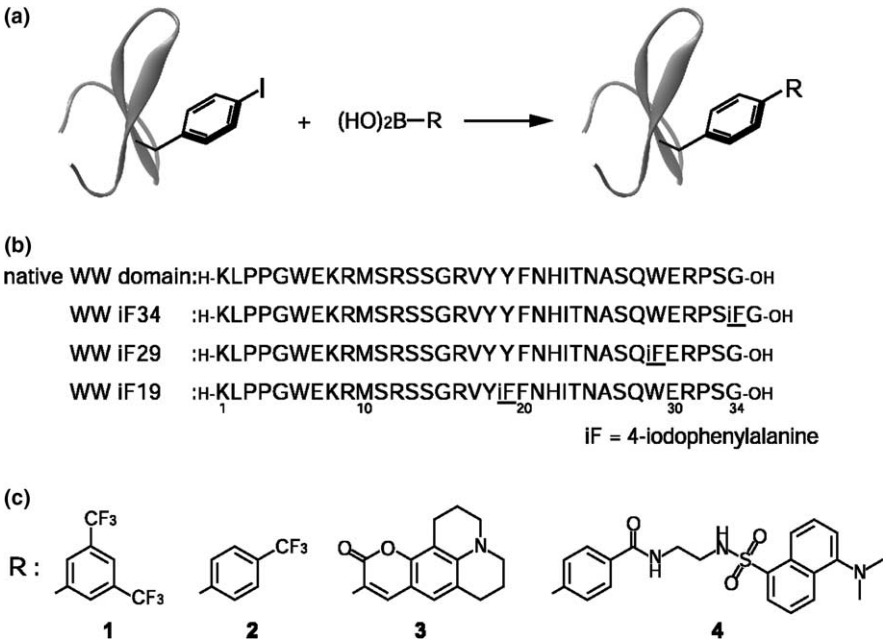

Scheme 1. (a) Schematic illustration of Suzuki-coupling on the protein surface of WW domain. (b) Amino acid sequences of the native WW domain and iodophenylalanine-appended WW domains (WW iFs). (c) Molecular structure of boronic acids used in this study.

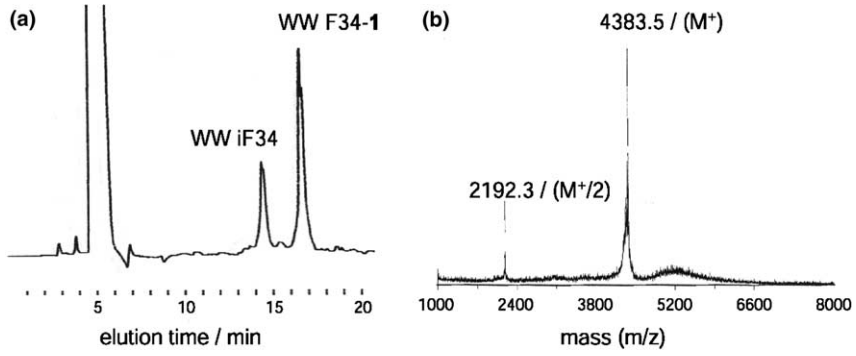

Figure 1. (a) HPLC analysis of Suzuki-coupling reaction of WW iF34 with **1** (entry 5). The peaks of WW iF34 and the coupling product WW F34-**1** are observed at 14.5 and 16.6 min, respectively. (b) MALDI-TOFMS (4383.5) of WW F34-**1** after HPLC purification.

product, it is clear that the reaction occurred selectively between 4-iodophenylalanine site of WW iF34 and **1**.

Table 1 summarizes the reaction conditions and yields for this reaction. Entries 1–3 show that the Suzuki-coupling proceeded in aqueous solution under the mild conditions for WW iF34. The higher temperature (40 °C) gave the better yield than the case of 25 °C. The palladium salt of the higher concentration (2.5 mM) did not considerably improve the yield, while the concentration of Pd^{II} lower than 1.0 mM decelerated the coupling reaction (data not shown). Thus, for the following entries (entries 4–10), the reaction temperature and the concentration of palladium salt were fixed at 40 °C and 1.0 mM, respectively. In the presence of 1% w/v *n*-octyl D-glucopyranoside (OG), which is frequently used for solubilizing membrane proteins (entry 4),[10] the reaction also proceeded with the improved yield, suggesting that Suzuki-coupling may be applicable to membrane proteins. Among entries 1–4, phenylalanine-appended WW domain (WW F34), a reduced byproduct of WW iF34, and/or precipitates derived from WW domain were also observed. However, the addition of 10% glycerol, which is a stabilizer generally used for many proteins suppressed such byproducts and precipitates so that over 70% yield was obtained (entry 5). Thus, it is concluded that the condition of entry 5 would be optimal. Other boronic acid, 4-(trifluoromethyl)phenylboronic acid (**2**), was also reactive under the similar conditions to that of the substrate **1**. Entries 6–8 revealed that Suzuki-coupling reaction of other WW mutants that possess a iodophenylalanine at the different position with **2** also proceeded with over 50% yield, showing that the reaction position in WW domain (i.e., the site of iodophenylalanine) is not a significant controlling factor. In addition to simple boronic acids such as **1** and **2**, fluorophore-appended boronic acids, **3** and **4**, were able to react to introduce the corresponding fluorophore to WW domain as shown in entries 9 and 10. These results indicate that various boronic acids and many reaction

Table 1. Suzuki-coupling reaction of WW iFs with **1**, **2**, **3**, and **4**

Entry	WW iF[a]	Boronic acid (mM)	Na_2PdCl_4 (mM)	Additive	Temp (°C)	Time (h)	Yield (%)[b]
1	iF34	**1** (5.0)	1.0	—	25	18	40
2	iF34	**1** (5.0)	1.0	—	40	20	38–50[c]
3	iF34	**1** (5.0)	2.5	—	40	18	40–53[c]
4	iF34	**1** (5.0)	1.0	1.0% w/v OG	40	18	58–77[c]
5	iF34	**1** (5.0)	1.0	10% v/v glycerol	40	18	74
6	iF34	**2** (3.0)	1.0	50% v/v glycerol[d]	40	18	~57
7	iF29	**2** (3.0)	1.0	10% v/v glycerol	40	18	58
8	iF19	**2** (3.0)	1.0	10% v/v glycerol	40	18	52
9	iF19	**3** (3.0)	1.0	50% v/v glycerol[e]	40	20	65
10	iF19	**4** (3.0)	1.0	50% v/v glycerol[e]	40	20	>90

[a] The concentration of WW iFs was fixed at 1.0 mM.
[b] Yields were calculated from HPLC peak area values as the average of three times experiments.
[c] Since the precipitation was observed in reaction mixture, yields of these entries were described with range.
[d] In the case of 10% v/v glycerol, yield could not correctly be determined due to broadening of HPLC peak.
[e] **3** and **4** were not completely soluble in 10% v/v glycerol/Tris–HCl buffer.

sites directed by iodophenylalanine may be available for Suzuki-coupling reaction on a protein surface.

Since we carried out Suzuki-coupling reaction under the mild conditions, the modified protein is expected to maintain its native structure and function during the reaction. The structure and function of the modified WW domain were investigated by circular dichroism (CD) spectroscopy and fluorescence anisotropy titration. In CD spectra, both WW iF19, a Suzuki-coupling substrate, and the WW F19-**2**, a Suzuki-coupling product, showed a positive CD maximum at 230 nm characteristic of folded WW domains, and a negative CD peak around 216 nm due to a typical of β-sheet structure as shown in Figure 2. These CD spectral features are similar to those of native WW domain.[11] Thus, it is clear that WW F19-**2** maintains the native-like folded structure after the coupling reaction. On the other hand, the positive peak at 230 nm disappeared after the coupling reaction in the cases of WW iF29, WW iF34, and other WW domain derivatives (WW iF2, WW iF6, and WW iF20) although they showed the positive CD peak at 230 nm before coupling reaction. These suggest that a suitable selection of the coupling site is crucial for the maintenance of the native-like protein structure.

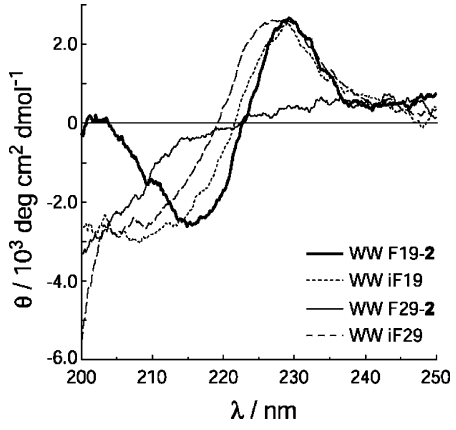

Figure 2. Circular dichroism spectra of WW F19-**2** (—), WW iF19 (----), WW F29-**2** (—), WW iF29(---) at 4 °C in 20 mM potassium phosphate buffer solution (pH 7.2, 100 mM NaCl).

It was established previously that Pin1 WW domain selectively binds proline-rich phosphorylated peptides such as CTD pS6,9 as shown in Figure 3.[12] Thus, the function of the present modified WW domain protein was evaluated by the phosphopeptide binding assay using fluorescent anisotropy experiment. Figure 3a shows the titration curve of the tetramethylrhodamine (TMR)-modified CTD peptides (pS6,9 and no-pS) with the modified WW F19-**2** domain. When TMR-CTD pS6,9 peptide was titrated with WW F19-**2**, the anisotropy value (r) determined by the anisotropic emission due to TMR[13] was gradually increased with a saturation manner. The titration curve thus obtained gave us the binding affinity of WW F19-**2** with TMR-CTD pS6,9 ($9.3 \times 10^3\ M^{-1}$). The value is almost same as that for native WW domain ($2.9 \times 10^4\ M^{-1}$). In contrast, no significant increase of the anisotropy value was observed when WW F19-**2** was added to TMR-CTD no-pS, indicating that WW F19-**2** does not bind to TMR-CTD no-pS. Thus, it is clear that WW F19-**2** retains the substrate selectivity even after Suzuki-coupling, that is, WW F19-**2** is capable of distinguishing phosphorylated peptide from nonphosphorylated one.

Interestingly, we found that the fluorescence of the coumarin-appended WW domain, WW F19-**3**, changed upon the addition of phosphorylated CTD peptides. Figure 3b and c show the fluorescence spectral change and the titration curve of the WW F19-**3** with CTD peptides, respectively. The fluorescence intensity at 510 nm due to the coumarin fluorophore decreased with the addition of CTD pS6,9 in a typical saturation manner. In the case of mono-phosphorylated substrate, CTD pS6, the fluorescence intensity decreased rather moderately compared to that of CTD pS6,9 and the fluorescence was slightly lessened for CTD no-pS peptide addition. From the titration curve, the binding constants of WW F19-**3** toward CTD pS6,9 and CTD pS6 were estimated as 3.9×10^4 and $3.7 \times 10^3\ M^{-1}$ by analyzing with the nonlinear least-squares curve fitting method[14] using Kaleida Graph (Synergy Software), respectively. These values were well consistent with those of native WW domain ($2.9 \times 10^4\ M^{-1}$ for CTD pS6,9 and $9.1 \times 10^3\ M^{-1}$ for CTD pS6). Therefore, it is

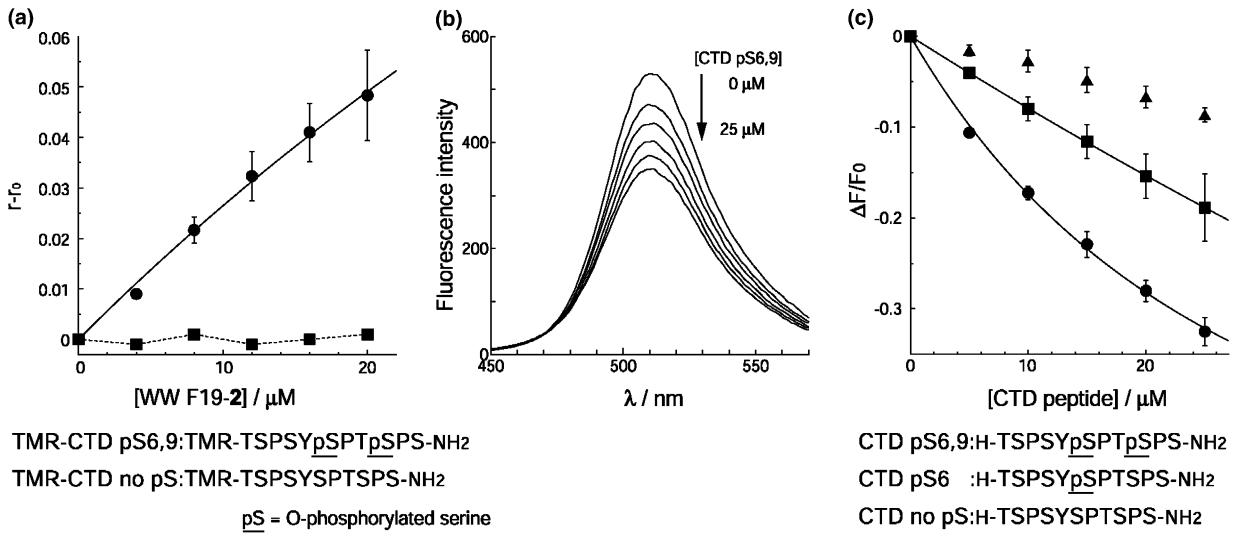

Figure 3. (a) Fluorescence anisotropy assay for the binding of WW F19-**2** to TMR-CTD peptides. TMR-CTD pS6,9 (●) or TMR-CTD no-pS (■) were titrated with WW F19-**2**: [TMR-CTD peptide] = 5.0 μM at 20 °C in 50 mM HEPES buffer (pH 7.2). The solid line was obtained with nonlinear least-squares curve fitting analysis for TMR-CTD pS6,9. (b) Fluorescence spectral change of WW F19-**3** (5.0 μM) upon the addition of CTD pS6,9: [CTD pS6,9] = 5.0, 10, 15, 20, and 25 μM at 10 °C in 10 mM HEPES buffer (pH 7.2, containing 2.0% v/v trifluoroethanol), λ_{ex} = 374 nm. (c) Fluorescence titration curve of WW F19-**3** (λ_{em} = 510 nm) with CTD pS6,9 (●), CTD pS6 (■), and CTD no-pS (▲). ΔF and F_0 represent decrement of fluorescent intensity and initial fluorescent intensity, respectively. The solid lines were obtained with nonlinear least-squares curve fitting analysis for CTD pS6,9 and CTD pS6.

demonstrated that WW F19-**3** produced by Suzuki-coupling was a fluorescent biosensor selective to phosphorylated peptides.

In conclusion, first demonstrated that the Suzuki-coupling reaction can be applied to the protein surface modification in aqueous solution. Our results showed that the reaction smoothly proceeds under ligandless mild conditions and that glycerol is a useful additive to suppress the side reaction so as to improve the reaction yield. Importantly, a WW domain modified through Suzuki-coupling retained its native structure and the selective binding ability for phosphorylated peptide thanks to the mild conversion conditions. It is also note-worthy that the modified fluorescent WW domain bearing a coumarin moiety acts as a fluorescent biosensor for phosphorylated peptides. Since the iodophenylalanine can be readily introduced into protein by extended codon method,[15] semisynthetic methods,[16] or others,[17] we envision that Suzuki-coupling reaction is widely applicable to various proteins in order to incorporate unnatural molecules with retaining natural functions.

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