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Affinity purification of the key enzyme of nyctinasty controlling the rhythm of leaf movement using gluconamidine ligand

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Abstract—Synthesis of affinity gel aimed for leaf-opening factor β-glucosidase (LOFG) and affinity purification of LOFG are presented. Gluconamidine-based β-glucosidase inhibitor was utilized for the ligand of the affinity gel. β-Glucosidase exhibiting activity shift throughout the day was selectively purified from *Lespedeza cuneata* Don by the affinity gel. The resulting LOFG exhibited a high substrate specificity toward the leaf-opening factor.

Leguminous plants are well known to close their leaves at night and open them in the morning. This rhythmic leaf movement is called nyctinasty whose rhythm is known to be controlled by a biological clock. In the past decade, a pair of leaf-movement factors, leaf-opening factor (LOF), and leaf-closing factor (LCF), was identified as chemical factors controlling leaf movement.[1,2] In the case of *Lespedeza cuneata*, potassium lespedezate (**1**) serves as LOF and potassium D-idarate (**2**) as LCF.[3,4] The endogenous level of **1** in the plant body is controlled by the metabolic enzyme, leaf-opening factor β-glucosidase (LOFG), whereas that of **2** remains nearly constant during the day (Fig. 1).[5] This diurnal change in the level of LOF in the plant body strongly affected the rhythm of nyctinasty. Thus, the rhythm of nyctinastic leaf movement is generated by the change in the LOFG activity during the day. Although this enzyme is considered as the key enzyme of nyctinasty, little is known about LOFG: Neither the circadian rhythmic activation mechanism nor the nature of LOFG was disclosed. Purification of LOFG should provide detailed information on these issues.

β-Glucosidase, which is a family of glycosidase, is involved in a number of important biological processes including storage and release of bioactive substances.[6,7] Therefore, purification methods for glycosidases have attracted the attention of biochemists. Affinity chromatography[8,9] has been widely used for this purpose. Some glycosides and their analogs, such as thioglycoside, were used as affinity ligands in the purification of glycosidase.[10,11] Glycosidase inhibitors, such as glycosylamidine,[12] deoxynojirimycin and its analogs[13,14] are also used as more effective affinity ligands. But in the affinity purification of substrate-specific glycosidase, they are sometimes insufficient ligands due to the lack of aglycon moieties. Recently we reported a concise synthesis of N-substituted gluconamidine, a strong and selective β-glucosidase inhibitor.[15,16] By this method, we synthesized gluconamidine (**3**) with the aglycon of LOF, which showed a high and specific inhibitory activity against β-glucosidase. Affinity chromatography using **3** as a ligand would allow selective purification of LOFG. In this Letter we report affinity purification and some aspect of the nature of LOFG.

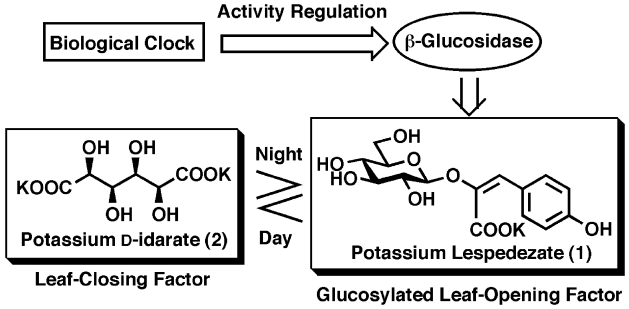

Figure 1. Leaf-movement factors (**1** and **2**) and circadian rhythmic control of the hydrolysis on **1**.

Keywords: Affinity chromatography; β-Glucosidase; Gluconamidine; Nyctinast.
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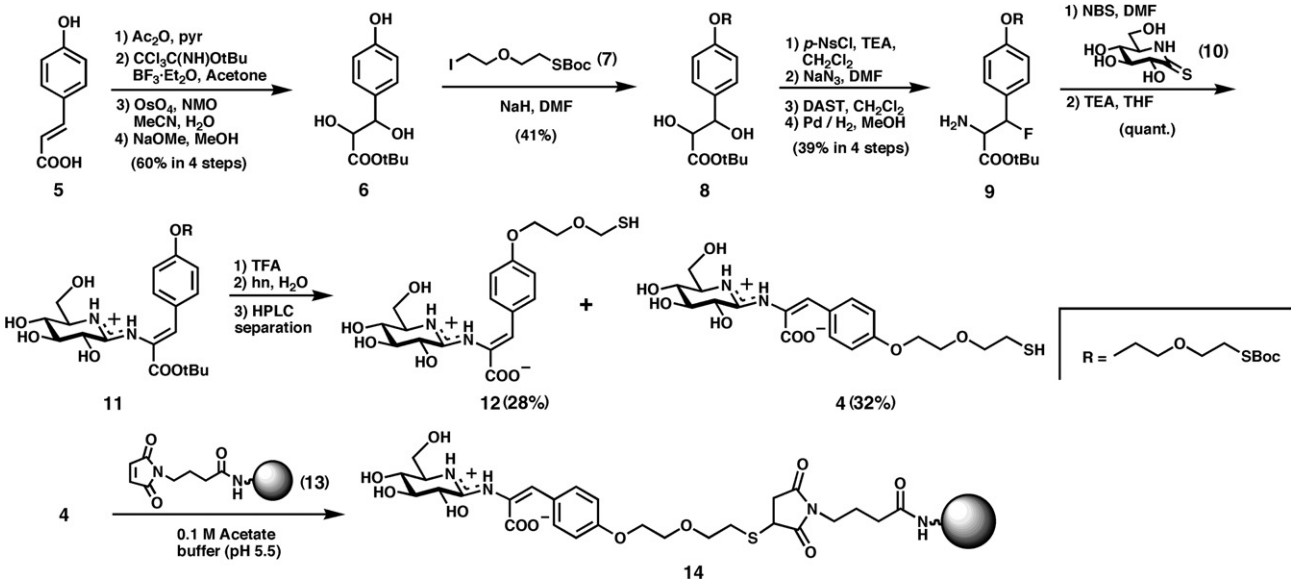
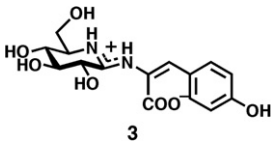
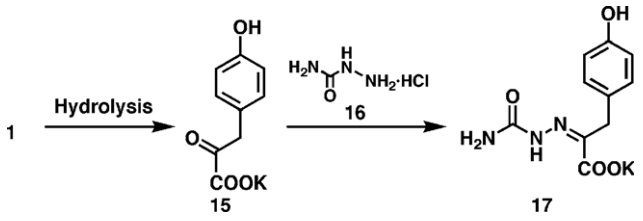


Scheme 1. Synthesis of affinity gel with gluconamidine ligand based on **1**.





Scheme 2. Derivatization of α-keto acids to semicarbazone for quantitative analysis of β-glucosidase activity.

Synthesis of affinity gel is shown in Scheme 1. From our preliminary study, LOFG was predicted to have a high substrate specificity and strictly recognizes the aglycon moiety of **1** (data not shown). Hence, we synthesized **4** which has a linker moiety on the phenolic hydroxy group connecting **3** with an affinity gel. The direct introduction of the linker moiety to **3** resulted in failure due to instability of the amidine moiety under coupling condition. Thus, the linker must be introduced before the formation of the amidine moiety. *p*-Hydroxycinnamic acid (**5**) was fully protected and diol was introduced to the olefin moiety by osmium tetroxide. After deprotection, the resulting phenol compound (**6**) was coupled with linker (**7**) containing a protected thiol group to give **8**. Then, the α and β-hydroxyl groups were substituted by azide and fluoride, respectively. Following reduction of the azide gave **9**, which was coupled with thionolactam (**10**)[17,18] by NBS to give **11**. Successive deprotection and photoisomerization by UV light (312 nm) gave a 1:1 mixture of **4** and **12**. Desired **4**[19] was obtained by purification using HPLC and then coupled with gel **13** containing a maleimide moiety, which was prepared from the commercially available Toyopearl AF-Amino-650M (Tosoh Co.) by the reported procedure,[12] to give affinity gel **14**. Performance of **14** was tested by commercially available β-glucosidase from *Aspergillus niger* (Fluka Co.). β-Glucosidase (0.1 unit) dissolved in 50 mM acetate buffer (pH 5.0) was applied on a column (∅ 5 × 100 mm) with 2 mL of affinity gel **14**. After washing the column with 50 mM acetate buffer (pH 5.0), the trapped β-glucosidase was eluted with the same buffer containing 2.0 M D-glucose (10 mL). Eleven percent of initial β-glucosidase activity (1.1×10^{-2} unit) was eluted by D-glucose, and the rest was recovered from the pass-through fraction. Then the capacity of gel **14** was estimated at 5.5×10^{-3} unit/mL gel. From this result, it was revealed that gel **14** can be used in the affinity purification of β-glucosidase (Scheme 2).

Affinity purification of LOFG from *L. cuneata* by **14** was then performed. Our previous study showed that LOFG activity in the plant body was monitored around evening.[5] Thus, we examined when the LOFG is activated. A series of acetone powder was prepared from the leaves of *L. cuneata* collected every two hours from 10:00 to 20:00. Each acetone powder was extracted by 0.1 M Bis–Tris buffer (pH 7.0), and the β-glucosidase activity of each extract was measured using **1** as a substrate. However, the quantitative analysis of β-glucosidase activity using **1** was very difficult because α-keto acid **15**, a hydrolytic product of **1**, was unstable and easily decomposed in aqueous solution. We thus developed a novel method for the quantitative analysis of unstable α-keto acids. Coupling of an α-keto acid with semicarbazide hydrochloride (**16**) gave stable semicarbazone (**17**).[20] Thus, instead of **15**, we analyzed **17** which is formed by the in situ coupling of **15** and **16** in aqueous solution.[21] By using this novel method, a remarkable increase in LOFG activity using **1** as substrate was observed between 14:00 and 16:00 (Fig. 2). We then used acetone powder prepared from the plants that were collected between 14:00 and 16:00 for further purification.

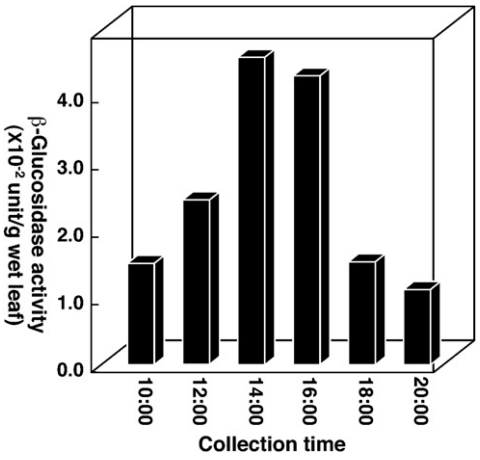


Figure 2. Diurnal change in the β-glucosidase activity in the leaves of *Lespedeza cuneata* L.

Table 1. Relative activity of LOFG against various substrates: activity was evaluated by analyzing corresponding aglycon by HPLC or colorimetry with *p*-nitrophenol

Entry	Substrate	Relative activity
1	Potassium lespedezate (**1**)	100
2	Potassium isolespedezate (**18**)	0
3	Dihydro lespedezate (**19**)	26
4	Potassium galactolespedezate (**20**)	0
5	Potassium galactoisolespedezate (**21**)	0
6	*p*-Nitrophenyl-β-D-glucopyranoside	179
7	*p*-Nitrophenyl-α-D-glucopyranoside	0
8	*p*-Nitrophenyl-β-D-galactopyranoside	0
9	*p*-Nitrophenyl-α-D-galactopyranoside	0
10	X-Glucose (**22**)	0[a]
11	Benzyl-β-D-glucopyranoside (**23**)	49

[a] Blue precipitate not detected.

The acetone powder was extracted with 0.1 M Bis–Tris buffer (pH 7.0) containing 1.0 M sodium chloride, 2 mM DTT, 5 mM EDTA, and 8% w/v Polyclar-VT (Wako Chemical Co.). After centrifugation, acetone was added to the supernatant to the extent of 60% v/v. The resulting precipitate was dissolved in 25 mM Bis–Tris buffer (pH 7.0) and then partially purified by HiPrep 16/10 Q XL (GE Healthcare Co.) anion exchange chromatography. All fractions with LOFG activity were then applied to an affinity column with gel **14**. After washing the gel with 25 mM acetate buffer (pH 5.0), LOFG was eluted by the same buffer containing 2.0 M D-glucose. However, no LOFG activity was observed in the fractions eluted by D-glucose. Then we used 10 mM aqueous solution of potassium lespedezate (**1**) as an eluent. The fraction eluted by **1** showed a strong LOFG activity of 1.6 unit/mg protein: 80-fold enhancement of the activity was achieved compared with the first extract (Table 2). Although **1** used for elution of LOFG was contained, the fraction was further purified by Superdex 200 10/300 GL (GE Healthcare Co.) to give LOFG which exhibited a 240-fold activity (4.8 unit/mg protein) compared with the first extract. The coincident elution of the protein (monitored at 280 nm) and glucosidase activity toward **1** was confirmed in this last step of purification (Fig. 3). The elution volumes of peaks in gel filtration corresponding to LOFG activity, β-glucosidase activity measured by using *p*-nitrophenylglucoside, and UV were all coincided. SDS-PAGE analysis of the resulting LOFG gave a smear band between 100 and 150 kDa (Fig. 4), whereas this LOFG gave a sharp single peak in the gel-filtration HPLC analysis using TSK-Gel SuperSW3000 (Tosoh Co.) (Fig. 4). The molecular weight of LOFG estimated from the elution volume in HPLC analysis corresponded to that from SDS-PAGE. This led us to presume that LOFG is subjected to some kinds of post translational modification because many of glycoproteins are known to exhibit a smear band on SDS-PAGE.[22–24] We thus examined the presence of the sugar chain. The modified method of O'Shannessy[25] was employed for detection of the sugar chain. The LOFG was subjected to SDS-PAGE and western blotting was performed on a PVDF membrane. The membrane was treated with 10 mM $NaIO_4$ and then by 0.1 mM biotinhydrazide. Chemiluminescence detection (ECL Advance™ Western Blotting Detection Kit, GE Healthcare Co.) with streptavidin-HRP conjugate clearly demonstrated the presence of the sugar chain on LOFG (Fig. 5). Dissociation of the sugar chain from LOFG and MS analysis of the sequence of resulting protein are now under way.

We also examined substrate specificity of LOFG. Activity of LOFG against some glycosides is seen Table 1. LOFG strictly differentiates the olefin moiety as shown in the comparison among potassium lespedezate (**1**), potassium isolespedezate (**18**) and **19** (entries 1–3). Potassium galactolespedezate (**20**) and potassium galactoisolespedezate (**21**), which have galactose instead of glucose, cannot be hydrolyzed (entries 4 and 5). Selectivity on the glycon moiety and stereochemistry of the glycosidic linkage were also investigated using a variety of *p*-nitrophenyl glycosides. LOFG specifically hydrolyzed β-glucoside (entries 6–9). Additionally, X-glucose (**22**) was not hydrolyzed at all (entry 10). However, a simple glycoside such as benzyl-β-D-glucopyranoside (**23**) was moderately hydrolyzed by LOFG (entry 11). These results showed that LOFG is a β-glucosidase, which

Table 2. Summary of purification of LOFG

Step	Total protein (mg)	Specific activity (unit/mg protein)	Purification (-fold)	Recovery (%)
Extract	16224.0	0.02	1.0	100
Acetone precipitation	4824.0	0.05	2.1	72.7
Anion exchange	197.0	0.15	6.5	7.9
Affinity	1.6	1.6	80.0	0.4
Gel filtration	0.1	4.8	240.0	0.1

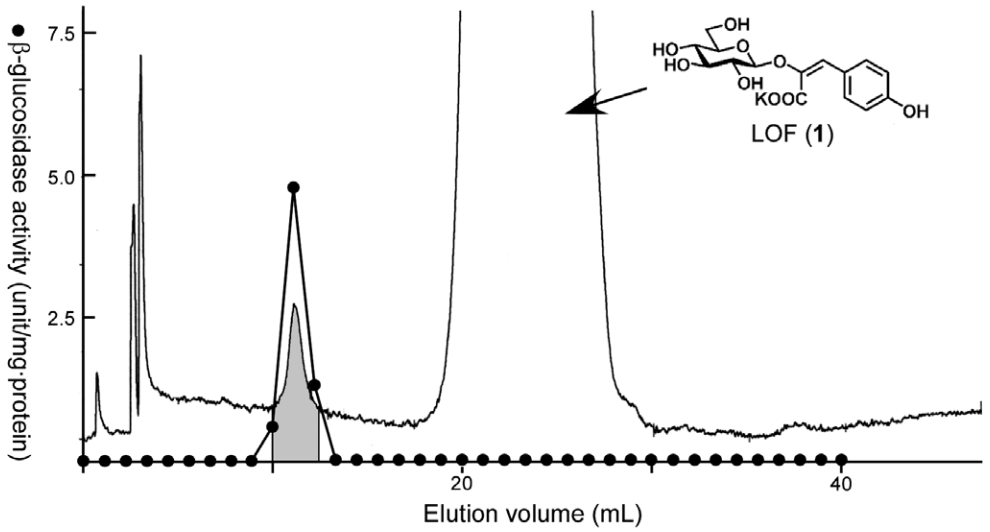


Figure 3. Final purification of LOFG: Column:Superdex 200 10/300 GL, Eluent: 25 mM Bis–Tris (pH 7.0), 100 mM sodium chloride, flow rate: 0.5 mL/min, detection: 280 nm. LOFG activity of each fraction was shown as black dot.

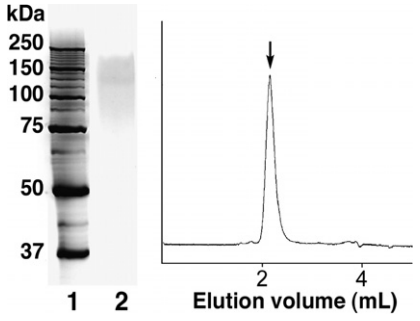


Figure 4. Purification of LOFG [right: SDS-PAGE analysis of LOFG (lane 1, Marker proteins; lane 2, LOFG); left: HPLC analysis of LOFG (column: TSK-Gel SuperSW3000; eluent: 50 mM phosphate buffer (pH 7.0), 150 mM sodium chloride; flow rate: 0.2 mL/min; detection: 215 nm)].

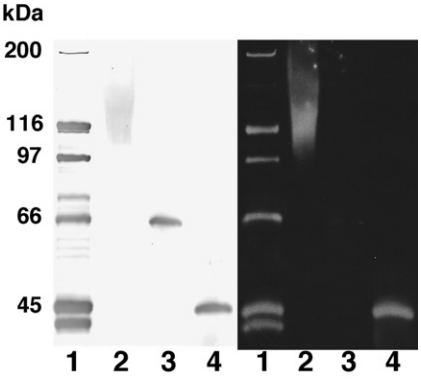
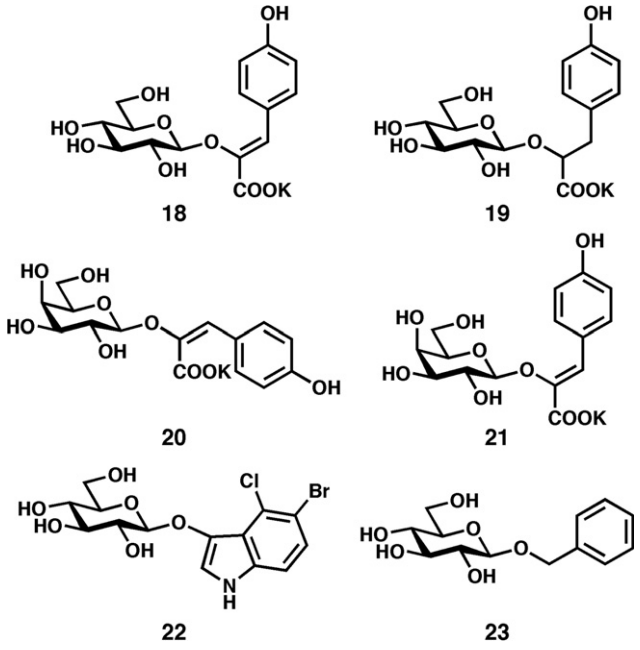


Figure 5. Detection of a sugar chain in LOFG. Right: membrane stained by colloidal gold total protein stain (Bio-Rad Lab.). Left: biotinylated sugar chain detected by chemiluminescence; lane 1, Marker proteins; lane 2, LOFG; lane 3, negative control (BSA); lane 4, positive control (Ovalbumin).

recognizes an aglycon moiety together with a glycon moiety, and showed a high selectivity toward **1**.



In conclusion, we have successfully purified LOFG, a key enzyme of nyctinasty, using affinity chromatography. Gluconamidine ligand **4**, which is designed on the structure of **1**, showed a strong inhibitory activity toward β-glucosidase, and was revealed to be quite effective for selective purification of LOFG. It was also shown that LOFG strongly recognizes an aglycon part of the substrate and exhibited a high substrate specificity toward **1**. These results strongly suggested that this enzyme is the LOFG which is involved in the diurnal control of the level of **1** in the plant body.

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19. Data of **4**: ^{1}H NMR (300 MHz, CD_3OD, rt): 7.54 (2H, d, $J = 8.7$ Hz), 6.90 (2H, d, $J = 8.7$ Hz), 7.22 (1H, s), 4.29 (1H, d, $J = 8.4$ Hz), 4.13 (2H, t, $J = 4.5$ Hz), 3.86 (1H, dd, $J = 2.4$, 11.4 Hz), 3.81 (2H, t, $J = 4.5$ Hz), 3.71–3.65 (3H, m), 3.65 (2H, t, $J = 6.6$ Hz), 3.44 (1H, m), 2.66 (2H, t, $J = 6.6$ Hz) ppm; ^{13}C NMR (75 MHz, CD_3OD, 25 °C): 167.4, 165.4, 162.1, 144.4, 134.3, 130.9, 126.3, 115.1, 74.24, 74.17, 70.3, 70.1, 69.6, 68.7, 62.6, 61.5, 24.7 ppm; HR ESIMS (positive): $[M+H]^+$ Found m/z 443.1481, $C_{19}H_{27}N_2O_8S^+$ requires m/z 443.1483; IR (film) ν: 3250, 1655, 1574, 1512, 1423 cm^{-1}; $[\alpha]_D^{19}$ −30.4 (c 0.10, MeOH). Spectrum data were compared with **3** to define stereochemistry of the olefin moiety.
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