

# A $\beta$ -Glucosidase Activity Potentially Involved in Cell Division and Wall Development of *Phyllostachys* Bamboo Suspension Cells

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## ABSTRACT

We propose a novel Madake (*Phyllostachys bambusoides*) bamboo suspension culture model for investigation of key enzyme(s) activity involved in growth/differentiation. Sedimented Cell Volume (SCV) and fresh weight (FW) of the suspension cultured cells reached 34% (v/v) and 8.7 g in 10  $\mu$ M 2,4-dichlorophenoxyacetic acid (2,4-D)-containing medium while only 7% (v/v) SCV and 1.9 g FW of the cells in 10  $\mu$ M gibberellic acid (GA<sub>3</sub>)-containing medium in 14 days. Proportion of mitotically active cells (S to G<sub>2</sub>/M phases) at a log phase was identified as 29.5% in the former cells with tiny cytoplasmic features while 5.4% in the latter cells with elongation, wall thickening, and lignification by using flow cytometry and laser scanning microscopic analysis. The total  $\beta$ -glucosidase (BGL) activity under the 2,4-D condition increased from 4.8 U in day 2 to 26.2 U in day 14 (ca. 5.5-fold) while a slight reduction, from 4.4 U in day 2 to 2.1 U in day 14 (ca. 0.5-fold), occurred when cell division was suppressed under the GA<sub>3</sub> condition. Ratio of the BGL activity of the soluble fractions to the membrane-associated fractions varied depending of the culture condition. The ratio was stable (2 to 8) during the culture period under the 2,4-D condition. Interestingly, the activity of the soluble enzyme fractions increased up to ca. 65% under the GA<sub>3</sub> condition in inverse proportion to the membrane-associated enzymes. All together, it was strongly suggested that the detected specificity/variability of BGL activity is potentially involved in cell division and lignification in Madake bamboo cells.

**Keywords:** Bamboo;  $\beta$ -Glucosidase; Cell Wall Development; *Phyllostachys bambusoides*; Suspension Culture

## 1. Introduction

The genus *Phyllostachys* is one of the largest genera of bamboo distributed mainly in the East Asia. As bamboos are fast-growing plants and have high carbon fixation ability, they are expected as renewable resources for biomass production and a potential modulator of global environment [1]. With the aim of elucidating growth features such as cell division and wall development, we previously developed callus and suspension cell culture of *Phyllostachys* bamboo. The bamboo suspension cells had a high proliferation potential with abundant deposition of  $\beta$ -glucan compounds [2]. We also reported the growth and carbohydrates (CH) metabolic features of suspension cells of Hachiku (*P. nigra*) bamboo in comparison with rice Oc cells [3], and tobacco BY-2 cells [4]. The profiles of sucrose degradation and uptake of the resulted mono-saccharides, fructose and glucose, in the media indicated one of the evidences that bamboo cells have a unique

feature of CH metabolisms [5].

We firstly examined three CH (sucrose, maltose, and cellobiose)-degrading activity of a novel Madake (*P. bambusoides*) bamboo cultured cells in comparison with that of bamboo intact tissues, such as shoots, axillary shoots, leaves, nodes, and culms. As the bamboo cells showed a unique feature of cellobiose degradation (**Table 1**), we then focused on an activity of the enzyme,  $\beta$ -glucosidase (BGL), potentially responsible for its degradation. As reviewed by Cairns and Esen [6], BGLs play important roles in cell wall development, lignification,  $\beta$ -glucan turnover, phytohormone activation, defense, and release of aromatic compounds in plants. We established a contrastive medium condition, *i.e.* proliferation (modified half strength Murashige and Skoog [7] medium (m1/2MS) in the presence of 2,4-D) and lignification (m1/2MS in the presence of GA<sub>3</sub>), and identified proportion of BGL activity of the soluble and the membrane-associated enzyme fractions in our bamboo suspension cell culture model with monitoring the fre-

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**Table 1. CH-degrading activity in cells/tissues of *P. bambusoides*.**

Cells/tissues	DW/FD	CH-degrading activity ( $\mu\text{mol/h/mg DW}$ )		
		Sucrose	Maltose	Cellobiose
Suspension cells	0.070	3.4	2.5	4.1
Shoots (top)	0.10	9.7	0.53	1.3
Shoots (base)	0.090	11	0.71	2.0
Axillary shoots	0.45	0.81	0.26	0.86
Leaves (immature)	0.47	1.1	0.32	0.76
Leaves (mature)	0.53	0.99	0.29	0.21
Nodes	0.72	0.11	0.12	0.26
Culms	0.61	0.36	0.070	0.20

quency of division/differentiation/lignification patterns of the cells by using flow cytometry analysis and laser scanning microscopic imaging. Technical details of bamboo suspension culture model are discussed in this study.

## 2. Materials and Methods

### 2.1. Plant Materials

Plant tissues, *i.e.* shoots, axillary shoots, leaves, nodes, and culms, were collected from a field-grown and/or a cultivated madake bamboo (*P. bambusoides* Sieb. et Zucc.), frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Suspension cultured cells were also prepared at appropriate periods as described below and used as materials.

### 2.2. Carbohydrates (CH)-Degrading Activity Assay

Approximately 10 - 70 mg of freeze-dried powder of bamboo suspension cells/ tissues were suspended into 1 ml of 100 mM CH solutions (cellobiose, maltose, and sucrose) in each well of 12-well micro-plate, and incubated at  $25^{\circ}\text{C}$  for 24 hours. The reaction mixtures were centrifuged at  $10,000 \times g$  for 1 min, and the supernatants were filtered through a  $0.45 \mu\text{m}$  filter (Amicon Ultrafree-MC, Millipore, USA). The filtrates were then analyzed by HPLC as described by Ogita *et al.* [5] with modifications. Briefly, a Cosmosil Sugar-D column (4.5 mm i.d., 250 mm in length, Nacalai, Japan) was set at  $40^{\circ}\text{C}$  with 75% (v/v) acetonitrile at a flow rate of 1 ml/min. Cellobiose, maltose, and sucrose were monitored with a refractive index (RI) detector (RI-101, Shodex, Japan). The CH-degrading activity was calculated based on the remaining amount of cellobiose/maltose/sucrose ( $\mu\text{mol}$ ) in the supernatant of reaction mixture after 24 hour-reaction.

### 2.3. Suspension Culture

Callus and suspension cultures of *P. bambusoides* were

generated as described by Ogita [2]. Approximately 1 g FW of callus tissues was suspended in 100 ml of liquid modified half strength Murashige and Skoog [7] medium (m1/2MS) supplemented with  $10 \mu\text{M}$  2,4-dichlorophenoxyacetic acid (2,4-D) in a 300-ml flask. The flasks were placed on a rotary shaker at 100 rpm in the dark at  $25^{\circ}\text{C}$ . Suspension cells were generated within 3 weeks after the initial culture. A portion of the liquid suspension cells (ca. 2 - 4 ml) was transferred to the same medium at 2-week intervals.

### 2.4. Growth Regulation of Suspension Cells

A portion of the liquid suspension cells was suspended in 50 ml of fresh m1/2MS media supplemented with  $10 \mu\text{M}$  2,4-D or  $10 \mu\text{M}$  gibberellic acid ( $\text{GA}_3$ ) in 200-ml flasks at cell density of 4% (v/v) sedimented cell volume (SCV). The flasks were placed on a rotary shaker at 100 rpm in the dark at  $25^{\circ}\text{C}$ . Changes in the SCV value were monitored during the 14 days of culture period. The SCV was measured by holding a liquid suspension cells for 15 min in a 50 ml centrifugation tube graduated in milliliters [8].

### 2.5. Flow Cytometry (FCM)

The cultured suspension cells (ca. 1 g FW) were collected from each flasks, treated with 1 ml of a chopping buffer (50 mM Tris-HCl, 50 mM  $\text{NaHSO}_3$ , 1% (v/v) TritonX-100, 140 mM 2-mercaptoethanol), carefully minced with a blade, and filtered using a tube with cell strainer cap (REF352235, BD Falcon, USA) for extraction of nuclei. The resulted nuclei were stained with  $5 \mu\text{M}$  Sytox Green. On average, 10,000 nuclei were counted per sample with a flow cytometer (FACS Calibur HG, BD, USA).

### 2.6. Lignification Capacity of Bamboo Suspension Cells

Lignification capacity of bamboo suspension cells was monitored by phloroglucinol-HCl reaction [9].

### 2.7. Laser Scanning Microscopy (LSM)

The cultured suspension cells were fixed using 4% (v/v) glutaraldehyde solution in sodium phosphate buffer (0.1 M, pH 7.2), and dehydrated with the ethanol series (25%, 50%, 70%, 80%, 90%, and 100%). They were stained with 0.01% Safranin and observed using a LSM (LSM510 META, Zeiss, Germany) for characterization of the frequency of division and/or enlargement patterns of the cells.

### 2.8. $\beta$ -glucosidase (BGL) Activity Assay

The used media in each culture period were collected

from the flasks as soluble enzyme fraction 1. Bamboo cultured cells (2 g FW) were also collected and grounded in a mortar with liquid nitrogen, extracted with 10 ml of 10 mM potassium phosphate buffer (KPB, pH 6.0), and centrifuged ( $10,000 \times g$ , 10 min,  $4^\circ\text{C}$ ). The supernatants were designated as soluble enzyme fraction 2. To the resulted precipitates, 10 ml of an extraction buffer (10 mM KPB containing 2.5% (w/v) sodium *N*-lauroylsarcosinate, 100 mM NaCl, 1 mM EDTA, pH 6.0) was added and the mixture was homogenized on an ice with a pestle, and gently mixed (3 h,  $4^\circ\text{C}$ ). After centrifugation ( $10,000 \times g$ , 10 min,  $4^\circ\text{C}$ ), the supernatants collected were further centrifuged ( $10,000 \times g$ , 10 min,  $4^\circ\text{C}$ ). They were applied to acetone precipitation for 15 min. at  $-35^\circ\text{C}$ . The precipitates were collected by centrifugation ( $10,000 \times g$ , 10 min,  $4^\circ\text{C}$ ), and were re-suspended to 10 mM KPB (pH 6.0) as membrane-associated enzyme fraction 1. The residues were designated as membrane-associated enzyme fraction 2. The protein content of each sample was determined by using a protein assay kit (Bio-Rad, USA) according to the method by Bradford [10].

BGL activity was measured with a synthetic substrate, *p*-nitrophenyl  $\beta$ -D-glucopyranoside (pNPGlc) [11]. Reaction mixture containing 2  $\mu\text{l}$  of DMSO solution of 500 mM pNPGlc, 20  $\mu\text{l}$  of 1 M KPB (pH 6.0) and 158  $\mu\text{l}$  of distilled water, was pre-incubated at  $40^\circ\text{C}$  for 5 min. The reaction was initiated by adding each enzyme fraction (200  $\mu\text{l}$  in total volume), incubated at  $40^\circ\text{C}$  for 15 min, and then terminated by adding 20  $\mu\text{l}$  of 1 M Tris-HCl buffer (pH 8.0). The absorbance at 405 nm was measured, and the content of *p*-nitrophenol (pNP) in the reaction mixture was calculated using a standard curve prepared under the same conditions. One unit (U) of BGL activity was defined as the amount of enzyme that catalyzing to release 1  $\mu\text{mol}$  of pNP per min under the above assay condition.

### 3. Results and Discussion

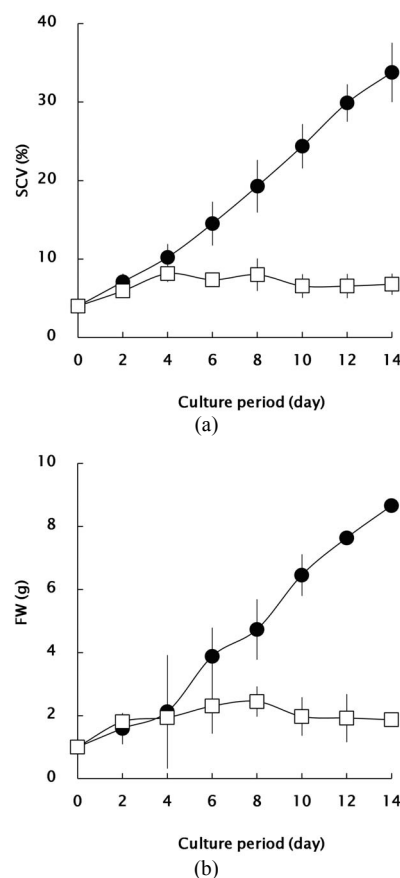
#### 3.1. CH-Degrading Activities in Bamboo

In order to assess CH-degrading activities of Madake bamboo, 100 mM CH solutions (sucrose, maltose, and cellobiose) were fed to the freeze-dried powder of various tissues and suspension cells, and decrease in the CH in 24 hours was measured by HPLC analysis. The results were summarized in **Table 1**. Sucrose, the major transport form of photo-assimilated carbon and a source of energy for plants, was highly degraded by shoots (9.7 - 11  $\mu\text{mol/h/mg}$  DW) and suspension cells (3.4  $\mu\text{mol/h/mg}$  DW). This feature is similar to the Hachiku bamboo cells [5]. The suspension cells could also degrade maltose, an important intermediate in the digestion of starch, at a late

of ca. 2.5  $\mu\text{mol/h/mg}$  DW. Interestingly, cellobiose, a partial form in the digestion of plant cell wall, was degraded by juvenile tissues like suspension cells (4.1  $\mu\text{mol/h/mg}$  DW) and shoots (1.3 - 2.0  $\mu\text{mol/h/mg}$  DW). Other mature tissues such as axillary shoots, leaves, nodes, and culms had weaker CH-degrading activity than suspension cells and shoots. Based on this finding, we speculated that an activity to degrade cellobiose will correlate with an early developmental process of bamboo cells.

#### 3.2. Growth Patterns of Suspension Cells

Since 2,4-D/GA<sub>3</sub> have co-stimulative/suppressive effects to cell division and wall development in *Coleus blumei* [12], they were independently applied to the bamboo suspension cells for regulation of the growth. Changes in SCV and FW during the culture period were monitored as shown in **Figures 1(a)** and **(b)**. Bamboo suspension cells in 2,4-D-containing medium showed a typical growth



**Figure 1.** Growth profiles of Madake bamboo suspension cells in m1/2MS medium, containing 10  $\mu\text{M}$  2,4-D (closed circle) and 10  $\mu\text{M}$  GA<sub>3</sub> (open square). (a): SCV value was calculated as the percentage of sedimented cell volume (ml) per 50-ml medium; (b): Fresh weight (FW) of suspension cells. All data are expressed as mean  $\pm$  SE (n = 3).

pattern as previously documented [8]. There was a lag growth phase for ca. 6 days, after that, the suspension cells showed an active growth during culture period. The SCV and FW reached 34% (v/v) and 8.7 g, respectively, in the 2,4-D-containing medium while only 7% (v/v) and 1.9 g in GA<sub>3</sub>-containing medium in 14 days.

### 3.3. Mitotic Activity and Histological Features of Suspension Cells

Proportion of mitotically active cells (S to G<sub>2</sub>/M phases) was summarized in **Figure 2**. Under the 2,4-D condition mentioned above, mitotically active cells were estimated from 14.9% (day 0) to 29.5% (day 8). In contrast, only 5.3% cells (day 8) were detectable under the GA<sub>3</sub> condition.

In order to investigate the frequency of division/differentiation/lignification patterns of bamboo suspension cells, LSM observation with safranin staining was performed. Tiny cytoplasmic dense cells which have pearl bluish auto-fluorescent signal in the thin primary cell wall and strong orange signal in the nucleus at the log phase (day 8) as shown in **Figure 3(a)**. In contrast, elongation and wall thickening of cultured cells strongly signalized in orange were detectable under the GA<sub>3</sub>

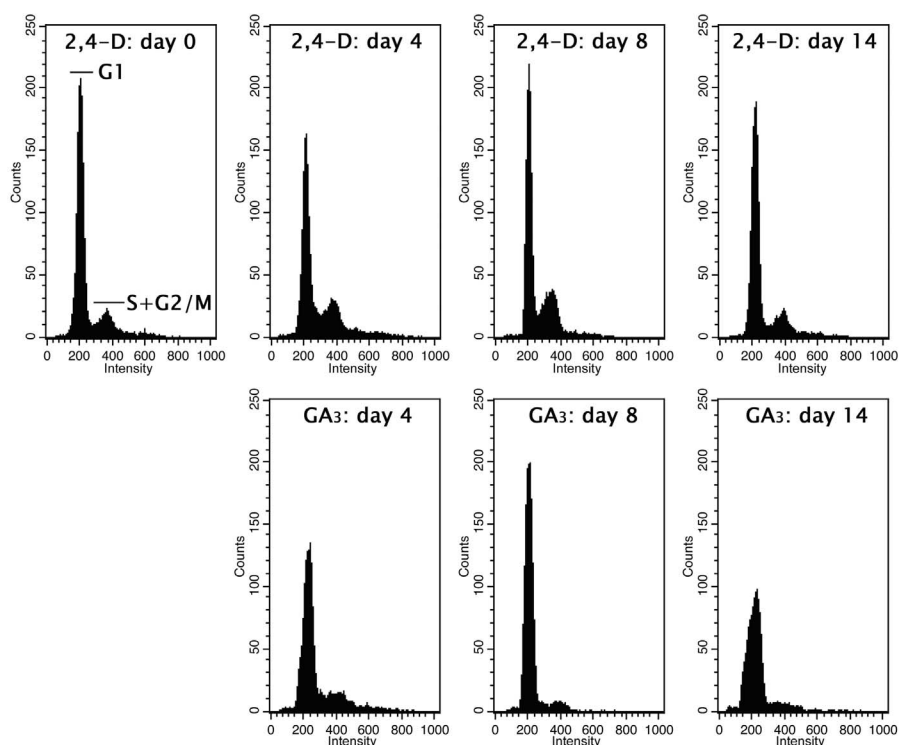
condition at day 8 (**Figure 3(b)**). Almost all cells cultured under the GA<sub>3</sub> condition elongated/enlarged at the end of the period (day 14). The resulted cells were subjected to phloroglucinol-HCl reaction to evaluate lignification pattern. As shown in **Figure 4**, the cells cultured under the GA<sub>3</sub> condition were stained with pale pink to red while any positive staining could not be detected in the cells cultured under the 2,4-D condition.

Gibberellin was known as a constituent for increasing fiber cell differentiation and secondary wall deposition in wheat (*Triticum aestivum* L.) culms [13]. It was also mentioned that the appearance to BGL activity coincided with lignification in *T. aestivum* suspension cells [14].

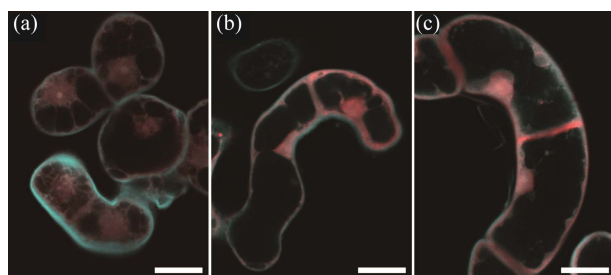
As our result indicates that proliferation/lignification were clearly controlled under the 2,4-D/GA<sub>3</sub> conditions in bamboo suspension cells, we applied this novel bamboo suspension cell culture model to evaluate the specificity/variability of BGL activity involved in division/differentiation/lignification.

### 3.4. BGL Activity of Suspension Cells

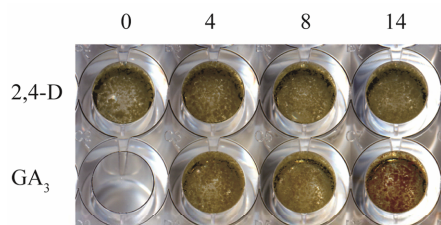
Total activity of BGL (**Figure 5(a)**) was expressed as the sum of soluble enzyme fractions 1 and 2 and membrane-associated enzyme fractions 1 and 2 (see Materials and



**Figure 2.** FCM analysis of Madake bamboo suspension cells. The bamboo suspension cells cultured in m1/2MS medium, containing 10  $\mu$ M 2,4-D and 10  $\mu$ M GA<sub>3</sub> were collected at day 0, 4, 8, and 14, respectively (See Figure 1). The parameters were set as follows. FSC detector (voltage: E00, amplified gain: 1.65, mode: log), SSC detector (voltage: 226, amplified gain: 1.00, mode: log), FL1 detector (voltage: 215, amplified gain: 1.00, mode: linear), FL1-A detector (amplified gain: 1.80, mode: linear), FL1-W detector (amplified gain: 3.00, mode: linear).



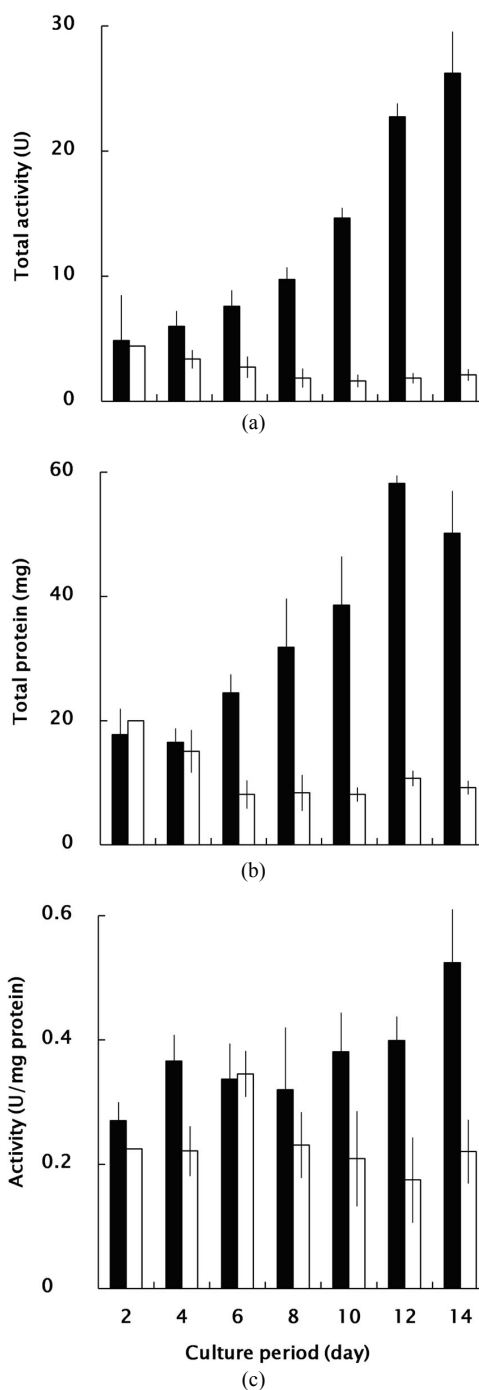
**Figure 3.** LSM imaging of Madake bamboo suspension cells stained with 0.01% Safranin. (a): Image of the cells cultured in m1/2MS medium containing 10  $\mu$ M 2,4-D (day 8); (b) and (c): Image of the cells cultured in m1/2MS medium, containing 10  $\mu$ M GA<sub>3</sub> (day 8 and 14), respectively. Images were acquired using plane scan mode. Scale bars = 20  $\mu$ m. The objective lens was a C-Apochromat 40  $\times$ /1.2 W corr. Channel specification was as follow: Plane scan: Blue diode laser 405 nm, 10.0%; Argon laser 488 nm, 5.0%; He-Ne laser 543 nm, 40.0%; Main Beam Splitter: HFT405/488/543/633; Beam Splitter 1: NFT 545; Beam Splitter 2: NFT 490; Filter; 550-625, BP 420-480 IR, BP505-530.



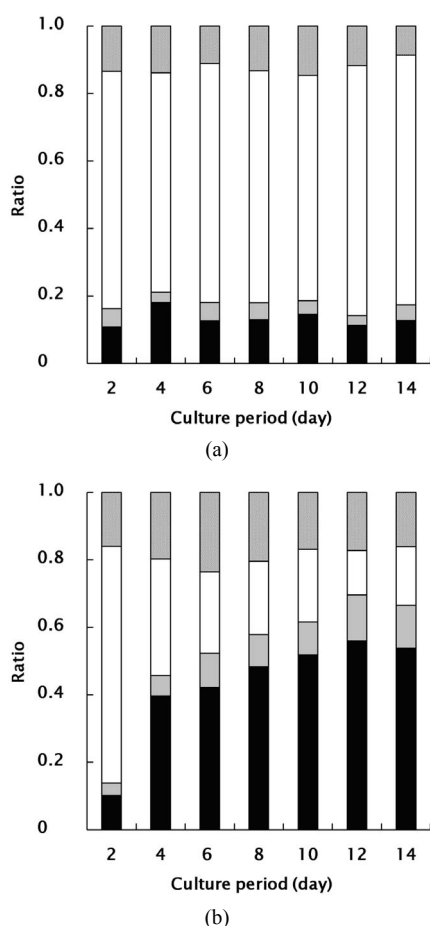
**Figure 4.** Lignification profiles of Madake bamboo suspension cells. The bamboo suspension cells cultured in m1/2MS medium, containing 10  $\mu$ M 2,4-D and 10  $\mu$ M GA<sub>3</sub> were collected at day 0, 4, 8, and 14, respectively (See Figure 2).

methods). The total activity of the cells grown under the 2,4-D condition increased from 4.8 U in day 2 to 26.2 U in day 14 (ca. 5.5-fold) while a slight reduction, from 4.4 U in day 2 to 2.1 U in day 14 (ca. 0.5-fold), occurred when cell division was suppressed under the GA<sub>3</sub> condition. The variation in the total protein which showed similar pattern with the total activity (**Figure 5(b)**) gave the specific activity of BGL. The activity slightly increased from 0.3 U/mg protein in day 2 to 0.5 U/mg protein in day 14 (ca. 1.9-fold) under the 2,4-D condition while a stable value (ca. 0.2 U/mg protein) was obtained under the GA<sub>3</sub> condition (**Figure 5(c)**).

The ratio of the enzyme activity of each fraction was also calculated as shown in **Figures 6(a)** and **(b)**. The ratio of the soluble enzyme fractions (1 and 2) to the membrane-associated enzyme fractions (1 and 2) under the 2,4-D condition was 2 to 8 during the culture period. The major activity was detected as the membrane-associated enzyme fraction 1 (ca. 70% of the total activity). On the other hand, activity of the soluble enzyme fractions (1 and 2), interestingly, increased up to ca. 65% in



**Figure 5.** Effect of 2,4-D and GA<sub>3</sub> on  $\beta$ -glucosidase activity of Madake bamboo suspension cells cultured in m1/2MS medium containing 10  $\mu$ M 2,4-D (closed bars) and m1/2MS medium containing 10  $\mu$ M GA<sub>3</sub> (open bars). The samples were collected at day 2, 4, 6, 8, 10, 12 and 14, respectively (See Figure 1). All data are expressed as mean  $\pm$  SE (n = 3). (a) and (b): Total activity of  $\beta$ -glucosidase, and total protein were expressed as the sum of soluble enzyme fractions 1 and 2 and membrane-associated enzyme fractions 1 and 2 (see Materials and methods); (c): Specific activity calculated from the data in (a) and (b).



**Figure 6.** The ratio of  $\beta$ -glucosidase activity of Madake bamboo suspension cells cultured in m1/2MS medium containing 10  $\mu$ M 2,4-D (a) and m1/2MS medium containing 10  $\mu$ M GA<sub>3</sub> (b). The samples were collected at day 2, 4, 6, 8, 10, 12 and 14, respectively (See Figure 1). Soluble enzyme fractions 1 (closed bars) and 2 (gray bars), and membrane-associated enzyme fractions 1 (open bars) and 2 (wavy gridded bars) (see Materials and methods).

total under the GA<sub>3</sub> condition in inverse proportion to the membrane-associated enzyme fractions (1 and 2) during the culture period. The major activity was detected as the soluble enzyme fraction 1 in the medium.

As supported by SCV, FW, FCM and LSM analysis (Figures 1-4), cell division was actively enhanced under 10  $\mu$ M 2,4-D condition and relatively high BGL activity could be seen (5.5-fold) in bamboo suspension cultured cells. The major BGL activity found in membrane associated enzyme fraction 1 (ca. 70% of the total activity), which could be solubilized in the presence of 2.5% (w/v) sodium *N*-lauroylsarcosinate, 100 mM NaCl, and 1 mM EDTA. In rice (*Oriza sativa*), for example, activities of cell wall-associated BGL during germination were characterized [15]. This enzyme appeared to be ionically bound to cell walls and was solubilized with 1 M NaCl

and its activity increased over 8-fold during germination. The functions of the enzyme were expected for hydrolysis and modification of oligosaccharides in cell walls of germinating rice seeds. As bamboo suspension cells showed abundant deposition of  $\beta$ -glucan compounds in cell wall during proliferation condition [2], another possibility was suggested that the presence of a  $\beta$ -glucanase with  $\beta$ -glucosidase activity like *Hordeum vulgare* [16].

Although the total BGL activity slightly reduced under the GA<sub>3</sub> condition as shown in Figure 5, distinct increasing in soluble BGL activity was detected (see Figure 6) in bamboo suspension cells. As 10  $\mu$ M GA<sub>3</sub> is a promotive phytohormone on lignification in bamboo suspension cells, it is expected that the major activity found in the soluble enzyme fraction 1 will contain a specific BGL for hydrolyzing monolignol glycosides. A coniferin BGL was identified from xylem tissue of *Pinus contorta* [17]. In phylogenetic analysis of *Arabidopsis thaliana* glycoside hydrolase family 1 was carried out and BGLU-45 and BGLU46 which categorized a cluster with *Pinus contorta* coniferin BGL were characterized [18].

Although we should conduct further investigations for isolation, characterization, and structure elucidation of expected BGL(s) from enzymological and genetic point of view, it is safe to conclude that the presented suspension cell culture model can be used as a tool for characterization of physiological and molecular biological events on cell division/differentiation/lignification of Madake bamboo with an efficient transformation protocol [19]. And such investigations are now in progress.

#### 4. Acknowledgements

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