Thermal Degradation Behavior of Ball-milled *Miscanthus* Plants and Its Relationship to Enzymatic Hydrolysis

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Correlations were determined between the thermal degradation behaviors of ball-milled *Miscanthus* plants and their enzymatic digestibilities. Overall, thermal degradation temperatures of *Miscanthus giganteus* were higher than those of *M. sinensis*. The differential thermogravimetric (DTG) curve of *M. giganteus* had a characteristic shoulder peak near 292 °C as opposed to that of *M. sinensis*. The thermal degradation temperatures of both ball-milled samples decreased with increased ball-milling time, although the composition was not changed by ball milling. Remarkable changes in the DTG curves of *M. sinensis* and *M. giganteus* occurred with ball milling for more than 60 min and 120 min, respectively. These thermal degradation results were similar to the results for physicochemical pretreatments and enzymatic digestibilities. The thermal decomposition temperatures of both ball-milled samples at 20% weight loss were most negatively correlated with the enzymatic digestibilities with a value of approximately -1.0.

Keywords: Thermal gravimetric analysis; Differential thermal gravimetric (DTG) curve; Miscanthus plants; Ball milling; Enzymatic hydrolysis

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INTRODUCTION

Lignocellulosic biomass consists of three main chemical components: cellulose, the most abundant organic material; lignin, the second-most abundant organic material; and hemicellulose, which mainly connects cellulose and lignin. Biorefinery processes that transform lignocellulosic biomass into useful fuels and chemicals are considered promising toward the establishment of a sustainable society and the reduction of carbon dioxide emissions. Recently, nanocellulose made from lignocellulose has attracted much attention as an advanced and environmentally friendly material (Yano 2010; Isogai *et al*. 2011). The isolation or separation of cellulose and other components is required to produce fuels (*e.g.*, bioethanol), chemicals, and nanocellulose.

Pretreatment techniques have been widely investigated for the facile production of fermentable sugars and nanofibrillated cellulose (Mosier *et al*. 2005; Pääkko *et al*. 2007; Hendriks and Zeeman 2008; Lagerwall *et al*. 2014; Hideno 2016). These pretreatments include: physical treatments, such as ball milling and wet-disk milling; physicochemical treatments, such as hot compressed-water treatment and steam explosion; chemical treatments, such as organosolv or sulfuric acid treatments; and biological treatments, such as pulping by white-rot fungi.

Ball milling, the most basic and efficient technique, has been investigated by many researchers, although this process normally consumes considerable energy (Sun and Cheng 2002; Hideno *et al*. 2009; Avolio *et al*. 2012). The high enzymatic digestibility of ball-milled samples has been explained through their increased surface areas and decreased crystallinity (Sun and Chen 2002; Mosier *et al.* 2005; Hendriks and Zeeman 2008).

Cellulases and hemicellulases are key enzymes in biorefinery processes such as fermentable sugars production (Himmel *et al.* 2007; Igarashi *et al.* 2011; Nonaka and Hideno 2014) and nanocellulose preparation (Pääkko *et al.* 2007) from pretreated biomass. Cellulases are enzymes that hydrolyze cellulose, and they generally consist of many enzyme molecules, including cellobiohydrolas I (CBH1), cellobiohydrolase II (CBHII), and endo-β-1,4 glucanase (EG).

Cellulase actions are strongly affected by the conditions and properties of the lignocellulosic biomass. Pretreatment processes such as ball milling and wet-disk milling are required to effectively hydrolyze lignocellulosic biomass by the cellulases (Himmel *et al.* 2007; Hendriks and Zeeman 2008). The pretreatment probably affects not only the enzymatic digestibility but also the thermal-degradation behavior. However, the relationships between the enzymatic digestibilities and thermal-degradation behaviors of the pretreated lignocellulosic biomass are not well known.

Thermogravimetric analysis (TGA) has been widely used to characterize the thermal-decomposition behaviors of plant biomass (Antal, Jr. and Varhegyi 1995; Negro *et al*. 2003). In general, TGA is applied to obtain calorimetric results in the development of fuels such as wood pellets. To effectively use plant biomass, it is necessary to develop it not only as a fuel for direct combustion, but also to convert it into value-added chemicals through non-combustion technologies such as enzymatic hydrolysis.

In a previous report, it was suggested that the thermal degradation characteristics of ball-milled Japanese cypress may be related to its enzymatic digestibility, although further details were unavailable. It was also suggested that the differential thermogravimetric curves (DTG) of pretreated biomass are strongly affected by their composition and condition (Hideno 2016). Moreover, based on DTG data, it was indicated that the effects of ball milling on non-cellulosic substances, such as hemicellulose and lignin, were more significant than those on crystalline cellulose. We also previously described the enzymatic hydrolysis of two ball-milled *Miscanthus* species (*M. giganteus* and *M. sinensis*) (Hideno *et al*. 2013), which are promising bioenergy crops with high biomass production potential because they are C4 plants (Clifton-Brown *et al.* 2004; Anzoua *et al*. 2011).

It has been reported that the effects of ball milling on enzymatic digestibility differ for *M. giganteus* and *M. sinensis*. However, a detailed understanding of these differences was not developed. Although thermal analyses of *Miscanthus* plants have been published (Szabo *et al*. 1996; Elmay *et al*. 2015), the relationships between their thermal degradation behaviors and other degradation properties, such as enzymatic digestibility, have not been described in detail.

In this study, *Miscanthus* plants were thermogravimetrically characterized, and the relationships between the thermal degradation properties of the ball-milled grasses and their enzymatic digestibility were investigated in detail.

EXPERIMENTAL

Materials

M. sinensis and *M. giganteus* were used similarly in the author's previous report (Hideno *et al*. 2013). Each sample was hand-cut to approximately 1 cm to 2 cm in length, and dry milled using a blender (ABSOLUTE3, Osaka Chemical Co., Ltd., Osaka, Japan). The dry-milled samples were electrically sieved (ANF-30; Nitto Kagaku Co., Ltd, Nagoya, Japan) for 30 min, and selected in size from 125 μm to 500 μm as the starting material.

Ball milling

Ball milling was performed using the same method described previously (Hideno *et al*. 2013). The sieved sample (approximately 3 g, 125 μm to 500 μm) and stainless balls (118 g) were placed in a stainless steel vessel, and ball milled at 400 rpm for 5 min to 240 min using a free-star ball-milling machine (Fritsch Japan Co., Yokohama, Japan), as shown in Fig. 1 (Hideno *et al*. 2013). These conditions were selected on the basis of our previous report.

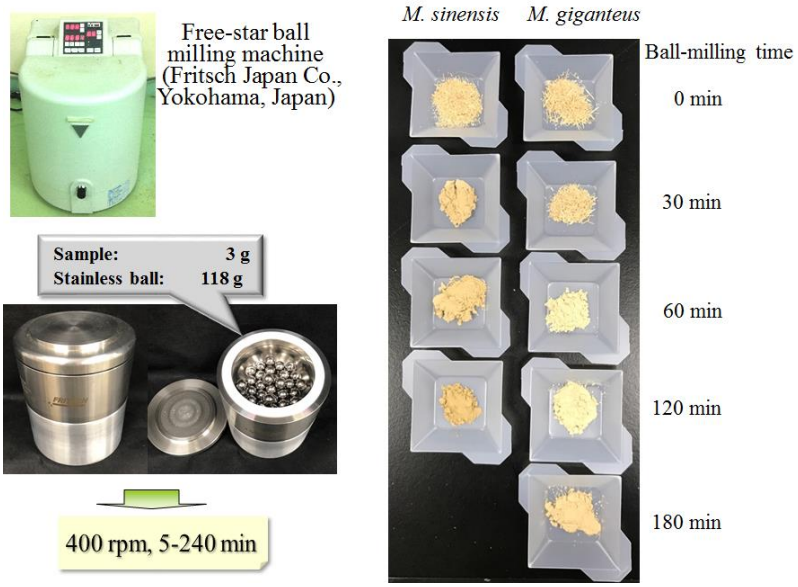

Fig. 1. Schematic diagram of ball milling and representative ball-milled samples. The figure was modified from our previous report.

Methods

TGA

The TGA of the original and ball-milled *Miscanthus* samples was conducted based on the previous report (Hideno 2016). The sample (approximately 5 mg) was pressed and formed into a tablet (Φ 4.5 mm) by a hand-press machine. The TGA instrument (TG/DTA6200; Seiko Instrument Co., Chiba, Japan) was used under a nitrogen atmosphere at a flow rate of 100 mL min^{-1} based on a previous report (Uetani *et al*. 2014) as follows; room temperature (RT) to 110 °C (40 °C min^{-1}), 110 °C for 10 min, 110 °C to 550 °C (10 °C min^{-1}), 550 °C for 10 min. This range of temperatures is typically used for detecting the

thermal degradation of cellulosic biomass. The thermogravimetric (TG) and DTG curves were plotted by calculations using Eqs. 1 and 2:

$$\text{TG (\%)} = (\text{thermal decomposition weight loss (g)} / \text{original weight (g)}) \times 100 \quad (1)$$

$$\text{DTG (\% min}^{-1}\text{)} = \text{TG (\%)} / \text{time for increase in temperature (min)} \quad (2)$$

The weight of the sample at 120 °C was defined as 100% dry weight. Curve fitting for the peak separation in the DTG curves was accomplished using TA7000 software (Hitachi High-Tech Science Co., version 10.41, Tokyo, Japan) combined with Fityk (Fityk, version 0.9.4, Warsaw, Poland). The split Gaussian method and the Levenberg-Marquardt algorithm were used for the peak separation and fitting of the DTG curves, respectively.

Relationships between thermal degradation properties and enzymatic hydrolysis of ball-milled Miscanthus plants

Enzymatic hydrolytic data for the ball-milled *Miscanthus* plants were obtained in part from a previous report (Hideno *et al*. 2013) or by using the method from that report. These data were obtained by triplicate experiments. The AP-treated *Miscanthus* plants (final concentration 5% w/v) and the Accellerase® 1500 (10 mg-protein g^{-1}; DuPont, Co., USA) were mixed well in the tube and they were incubated at approximately 50 °C for 72 h with agitation. The mixture was centrifuged at 20,000 g for 10 min. The supernatant was filtered out and subjected to high-pressure liquid chromatography equipped with an Aminex HPX-87P column and a Carbo-P micro-guard cartridge (BioRad, Hercules, CA, USA).

Correlation factors between the thermal degradation properties and enzymatic digestibility were calculated using Excel software (Microsoft Co., Excel 2013, Redmond, USA). Almost all of the ball-milled samples used in this study (*M. giganteus*: ball-milling times of 0, 30, 60, 120, and 180 min; *M. sinensis*: ball milled for 0, 30, and 60 min) were applied in the correlation factor calculations.

RESULTS AND DISCUSSION

Thermal Degradation Behavior of Raw and Ball-milled *Miscanthus* Plants

Judging from the TGA curves of the raw *M. giganteus* and *M. sinensis* (Fig. 2), the thermal decomposition of *M. sinensis* occurred at a lower temperature than that of *M. giganteus*. The thermal decomposition temperature of *M. giganteus* at 1% weight loss and the differential thermogravimetric peak were 225.5 °C and 341.5 °C, respectively, and were higher than those of *M. sinensis* at 209.6 °C and 327.2 °C, respectively. The DTG peaks at 341.5 °C and 327.2 °C were likely derived from the thermal degradation of cellulose. These differences in thermal degradation behavior between *M. sinensis* and *M. giganteus* are likely derived from their components. The cellulose, xylan, and lignin contents of *M. giganteus* are higher than those of *M. sinensis* (Hideno *et al*. 2013). Our results suggest that *M. giganteus* has more tolerance not only to physical and chemical pretreatment but also thermal degradation than *M. sinensis*.

The effects of ball milling on the DTG curves of the *Miscanthus* plants are shown in Fig. 3. Commonly observed features in the ball-milled samples were decreased peak heights and decreased thermal degradation temperatures, which shifted the DTG curves to the left.

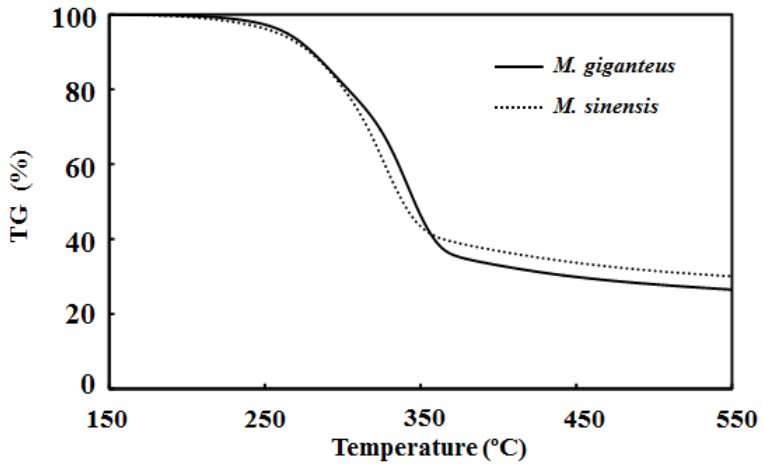

Fig. 2. TG curves for *M. giganteus* and *M. sinensis*

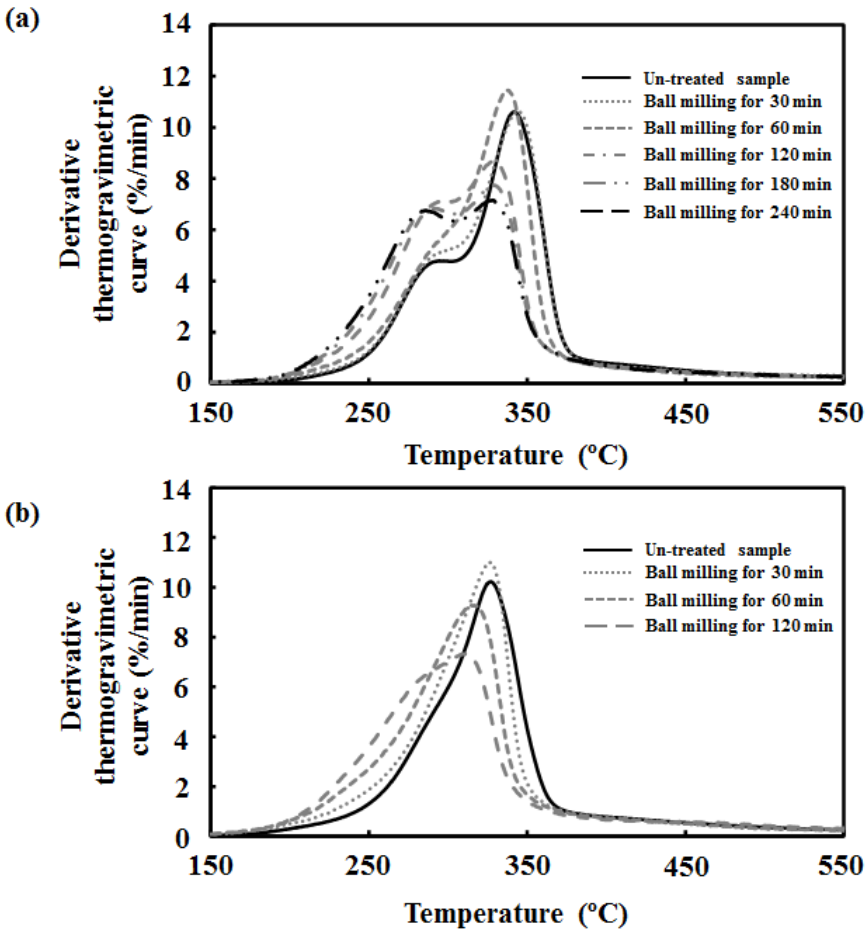

Fig. 3. Changes in DTG curves for (a) *M. giganteus* and (b) *M. sinensis* during ball milling

In a previous report (Hideno *et al*. 2013), we showed that the enzymatic digestibilities of *M. giganteus* and *M. sinensis* were significantly increased by ball milling for more than 120 min and 60 min, respectively. In this study, the DTG curves of *M. giganteus* and *M. sinensis* are remarkably altered and shifted to lower temperatures after more than 120 and 60 min of ball milling, respectively. Thus, for both *Miscanthus* plants, the changes in their DTG curves with respect to ball-milling time can be correlated with the changes in their enzymatic digestibilities with respect to ball-milling time. Interestingly, the characteristic DTG shoulder peak of *M. giganteus* remained, and its area increased after ball milling, although the thermal-decomposition temperatures at the shoulder DTG peak's top gradually decreased. For both samples, the DTG peak areas at lower temperatures (*i.e.*, below 300 °C) gradually increased with increased ball-milling time. The shoulder peaks near 292 °C are probably derived from a hemicellulose, such as xylan, as previously suggested (Werner *et al.* 2014). In our previous report, the DTG peaks of Avicel (microcrystalline cellulose) were hardly changed by ball milling, although the crystallinity was lost after ball milling for more than 20 min (Hideno 2016). In contrast, the DTG peaks of both *Miscanthus* plants were remarkably affected by ball milling. This tendency of *Miscanthus* plants was the same as observed for ball-milled Japanese cypress (Hideno 2016). It is likely that not only crystalline cellulose but also portions of the hemicellulose and lignin components in the *Miscanthus* plants were broken and denatured by ball milling.

Separated and Curve-fitted DTG Curves of *M. giganteus* and *M. sinensis*

The DTG curves and curve-fitted DTG curves were compared for raw and ball-milled *M. giganteus* and *M. sinensis* (Fig. 4). These ball-milled samples were selected based on the results of the previous report (Hideno *et al.* 2013), which had shown remarkably improved enzymatic digestibility. Separating and curve-fitting the DTG peaks indicated that the DTG curves of the *Miscanthus* plants comprised of two main peaks with several trace peaks. As mentioned, *M. giganteus* had a characteristic DTG shoulder peak at approximately 292 °C while *M. sinensis* did not (Fig. 3). However, curve-fitting the DTG data for *M. sinensis* revealed a similar peak near 296 °C (Fig. 4 (b)), which was equivalent to the DTG shoulder peak of *M. giganteus*. In general, DTG curves derived from the thermal decomposition of xylan include two peaks: a small shoulder peak at approximately 230 °C, and a main peak at approximately 290 °C (Yi-Min *et al*. 2009; Collard and Blin 2014). The separated peaks at 292 °C (Fig. 4(a)) and 296 °C (Fig. 4(b)) were probably the main peak attributed to the thermal decomposition of xylan as speculated earlier. The author's results indicated that the DTG peak derived from xylan in *M. sinensis* was detected by separating and curve-fitting. Comparing the constituent sugars of both species, the glucose and xylose contents derived mainly from the cellulose and xylan in *M. giganteus* are higher than those in *M. sinensis* (Hideno *et al*. 2013). However, the ratio of xylan/cellulose in *M. giganteus* of approximately 0.45 was lower than that of *M. sinensis* (0.48). It may be possible to correlate not only the xylan content, but also the bonds between cellulose and xylan, with the DTG shoulder peak at approximately 292 °C to 296 °C. The area of each highest curve-fitted DTG peak could be correlated with the cellulose, similar to previous research results (Hideno 2016). As shown in Fig. 4, these main peaks gradually shifted to lower temperatures through ball milling, except for the separated peak at 301.7 °C in Fig. 4(c). The reason this peak shifted to higher temperature may have been due to the unexpected generation of chemical bonds between cellulose and hemicellulose

by ball milling. The resistance towards the physicochemical treatment of *M. giganteus* was higher than that of *M. sinensis* judging from the ball-milling results (Hideno *et al*. 2013). Indeed, the thermal degradation temperature of *M. giganteus* was higher than that of *M. sinensis*. This may be related to the percentages of cellulose, xylan, and lignin in the grass because their contents in *M. giganteus* are greater than those in *M. sinensis* (Hideno *et al*. 2013). In particular, the lignin content is important for the thermal degradation of crystalline cellulose based on the previous report (Hilbers *et al.* 2015). Alternatively, the amount of lignin and the bond strengths of the cellulose and hemicellulose may be discerned from the DTG curve. The author's results indicate that the DTG curve and curve-fitted DTG curve can be applied as a markers to classify or select the grass species.

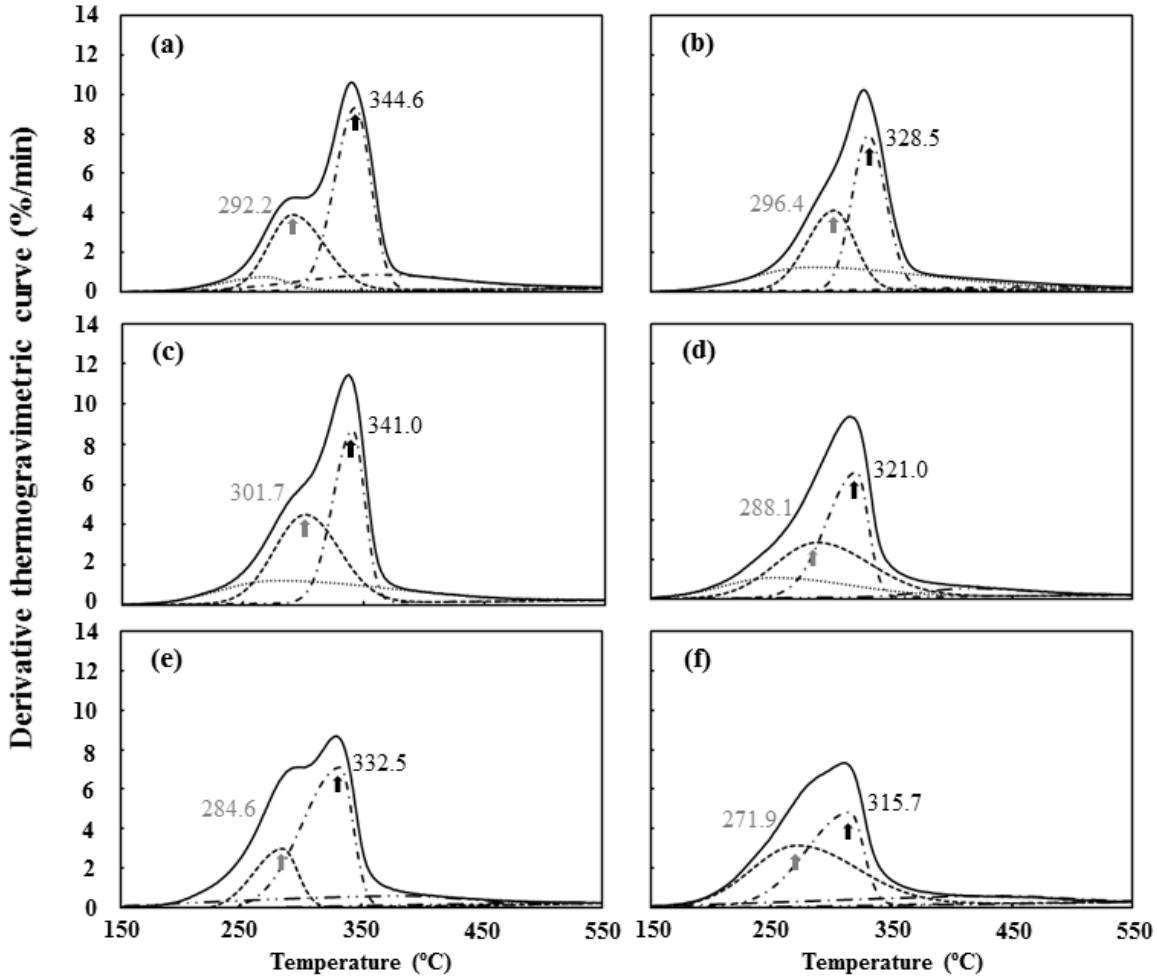

Fig. 4. Separated and curve-fitted DTG curves of (a) raw *M. giganteus*, (b) raw *M. sinensis*, (c) *M. giganteus* subjected to ball milling for 60 min, (d) *M. sinensis* subjected to ball milling for 60 min, (e) *M. giganteus* subjected to ball milling for 120 min, and (f) *M. sinensis* subjected to ball milling for 120 min

The solid and broken lines represent the original DTG curves, and the separated and curve-fitted DTG curves, respectively. The broken lines indicate the calculated virtual lines. The dashed lines, and dotted-and-dashed lines represent the assumed DTG curves of xylan and cellulose, respectively.

Effect of Ball Milling on Thermal Degradation Temperatures of *M. giganteus* and *M. sinensis*

Changes in the thermal-decomposition temperatures of the ball-milled samples for each milling time are shown in Fig. 5. All thermal-decomposition temperatures of *M. giganteus* were higher than those of *M. sinensis*. In a previous report, *M. giganteus* was more resistant to physical and chemical treatments and enzymatic degradation than *M. sinensis* (Hideno *et al*. 2013). The thermal degradation results herein display a trend similar to the results of the previous study. Comparing the thermal decomposition temperatures of the non-treated samples (ball-milling time of 0 min), the difference in the thermal-degradation temperature at 1% weight loss was the highest. This result and other reports (Hideno 2016) indicate that the temperature at 1% weight loss sensitively affected the physical and chemical properties of the materials. Increases in ball-milling time led to decreased thermal decomposition temperatures for all samples. In the author's previous report, the effects of ball milling on the thermal-decomposition temperature of microcrystalline cellulose were less than those on lignocellulosic materials such as Japanese cypress (Hideno 2016). In other words, the difference between the thermal-degradation temperatures of microcrystalline and amorphous cellulose was not remarkable although that of amorphous cellulose was slightly lower than that of microcrystalline cellulose. Almost all the differences in the thermal-decomposition temperatures resulting from ball milling were likely caused by damage to not only cellulose but also the hemicellulose and lignin. After ball milling for more than 120 min in *M. giganteus* and more than 60 min in *M. sinensis*, no further reductions in the thermal degradation temperatures were observed. These results agree with the trends observed for enzymatic digestibility in previous research (Hideno *et al*. 2013). The results in this study indicated that the changes in thermal degradation temperatures were correlated with the enzymatic digestibility in ball-milled samples.

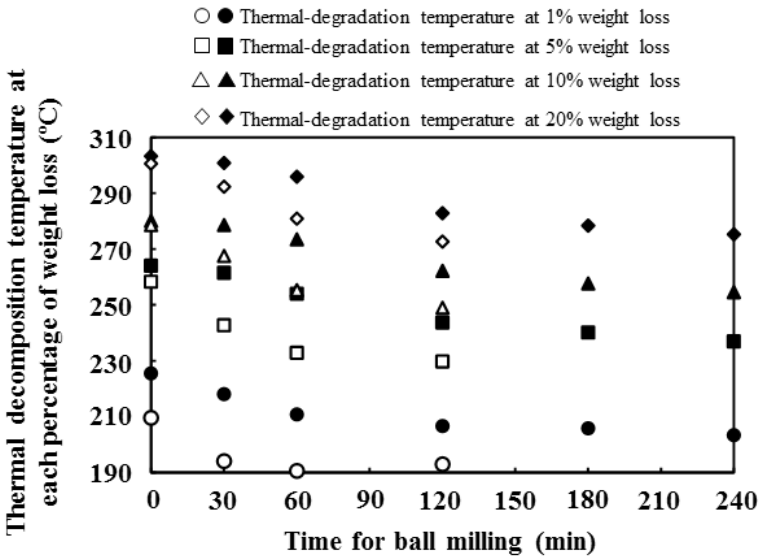

Fig. 5. Changes in thermal-degradation temperatures for each weight loss as a function of ball-milling time. Open and closed symbols represent the thermal degradation temperatures of (a) *M. giganteus* and (b) *M. sinensis*.

Relationships between Thermal-Degradation Properties and Enzymatic Hydrolysis of the Ball-milled *Miscanthus* Plants

Relationships between the percentage of weight loss during thermal decomposition, the correlation factors for enzymatic digestibility (glucose and xylose yields), and thermal decomposition temperatures are shown in Fig. 6(a). The correlation factors were calculated by using the combined data for the ball-milled *Miscanthus* plants (*M. giganteu*s and *M. sinensis*). The correlation factors for both the glucose and xylose yields and thermal degradation temperatures decreased with increasing weight loss, reaching nearly -1.0 at 20% weight loss. The relationship between the thermal- degradation temperature at 20% weight loss and the sugar yields by enzymatic hydrolysis are shown in Fig. 6(b). High linearity was observed for the glucose yield from 275 °C to 305 °C, and in the xylose yield from 275 °C to 300 °C. Correlation factors for the glucose and xylose yields were -0.98 (*p* value: 2.30×10^{-8}) and -1.00 (*p* value: 2.27×10^{-9}), respectively.

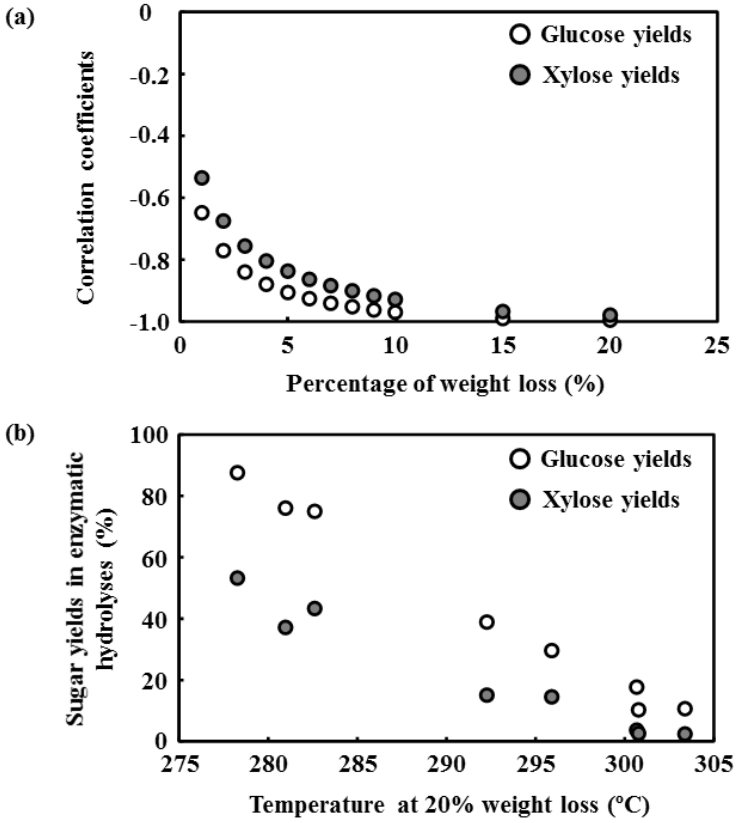

Fig. 6. Correlations between thermal-degradation properties and enzymatic digestibilities of ball-milled *Miscanthus* plants: (a) Relationships between percentage weight loss during thermal degradation and correlation coefficient in thermal-degradation temperatures and sugar yields in enzymatic hydrolysis of ball-milled samples; (b) Relationships between thermal-decomposition temperatures at 20% weight loss. The open and gray circles represent glucose and xylose yields, respectively, in the enzymatic hydrolysis of *Miscanthus* plants.

The relationships between the thermal-degradation temperatures and enzymatic digestibility were remarkably negative. These results suggest that enzymatic digestibility can be calculated by the thermal degradation temperature at 20% weight loss in ball-milled

herbaceous plants such as *Miscanthus*. In the range of temperatures wherein high linearity was obtained, the main events were the thermal decomposition of nearly all the xylan and the onset of the partial degradation of cellulose. The range of thermal-degradation temperatures at 20% weight loss closely corresponds with the DTG shoulder peaks of *M. giganteus* (Fig. 3) and the assumed DTG curves of xylan (Fig. 4). In other words, the enzymatic digestibility (glucose and xylose recoveries) of ball-milled *Miscanthus* plants mainly depended on the content and structure of the hemicelluloses such as xylan. In general, the effects of ball milling on increasing enzymatic digestibility and decreasing thermal-degradation temperatures has been explained on the basis of the increased surface area, decreased size, and decreased crystallinity of cellulose (Sun and Chen 2002; Mosier *et al.* 2005; Hendriks and Zeeman 2008; Kim *et al.* 2010; Khan *et al.* 2016). However, the addition of xylanase has also been reported to increase the yield of pentose and glucose from the ball-milled *M. sinensis* (Yoshida *et al.* 2008). The present results and this reference suggest that the structure changes and denaturing of xylan by ball milling also contribute to the increased enzymatic digestibility and decreased thermal-degradation temperature. In contrast, low linearity was observed in the relationship between the thermal degradation temperature at less than 5% weight loss and enzymatic digestibility, although there was a negative correlation. This was because the temperature required for 5% weight loss was relatively low and was insufficient for the thermal degradation of xylan, which was highly related to enzymatic digestibility. The author's results indicate that the enzymatic digestibility of the ball-milled *Miscanthus* plants can be estimated by the thermal-degradation temperature required for 20% weight loss. Additionally, the results indicate that the species and enzymatic digestibility of ball-milled *Miscanthus* plants can be estimated by thermogravimetric analysis.

CONCLUSIONS

To develop a simple evaluation method for distinguishing species of *Miscanthus* and estimating their enzymatic digestibility, thermogravimetric analyses of raw and ball-milled *M. giganteus* and *M. sinensis* were conducted, and the relationships between the thermal-degradation temperature and enzymatic digestibility of the ball-milled *Miscanthus* plants were investigated.

1. The thermal-degradation temperature of *M. sinensis* was lower than that of *M. giganteus* similar to the enzymatic hydrolysis results in the previous study.
2. A characteristic shoulder peak near 292 °C observed in the DTG curve of *M. giganteus* was retained in the ball-milled sample.
3. The thermal-degradation temperatures of both samples (*M. giganteus* and *M. sinensis*) decreased with increased ball-milling time, although the rate of decrease remained level beyond 60 min of ball milling.
4. The thermal-degradation temperature of ball-milled *Miscanthus* plants at more than 20% weight loss was negatively correlated with enzymatic digestibility, with a value of approximately -1.0.

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