# Effect of Tween 80 on Hydrolytic Activity and Substrate Accessibility of Carbohydrolase I (CBH I) from *Trichoderma viride*

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**ABSTRACT :** The present study examined the effects of Tween 80 on the attachment and hydrolytic activity of a cellulase enzyme against ball-milled cellulose (BMC), using the whole component (native CBH I) and the catalysis module (core CBH I) of carbohydrolase I purified from *Trichoderma viride* (Meicelase, Meiji Seika, Tokyo, Japan). The effects were evaluated as protein concentrations in the supernatant after mixing enzyme and substrate with Tween 80 at room temperature. Tween 80 decreased the adsorption of native CBH I and core CBH I onto BMC ($p<0.001$) and increased the amount of reducing sugars released from BMC by native CBH I ($p<0.001$). However, Tween 80 did not enhance the hydrolytic activity of core CBH I. Observations using SEM revealed that Tween 80 caused cellulose filter paper to swell and enhanced surface cracks and filaments caused by native CBH I but not by core CBH I. These results suggested that Tween 80 decreases enzyme adsorption to its substrate but enhances enzymatic activity. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 5 : 684-689)***

**Key Words :** Enzymatic Activity, Cellobiohydrolase, Tween 80

## INTRODUCTION

Surfactants enhance the enzymatic hydrolysis of cellulolytic material for industrial (Fendler and Fendler, 1975; Castanon and Wilke, 1981; Kim et al., 1982; Ooshima et al., 1986; Helle et al., 1993; Eriksson et al., 2002; Alkasrawi et al., 2003) and agricultural applications (Aksenova et al., 1994; Goto et al., 2003a, 2003b; Wang et al., 2003). Surfactants also increase ruminal cellulase activity (Kamade et al., 2000; Goto et al., 2003b; Lee and Ha, 2003), total numbers of bacteria *in vitro* (Goto et al., 2003a; Lee et al., 2003) and improve the efficiency of beef production (Wang et al., 2003).

A total mixed ration of dairy cattle treated with fibrolytic enzyme and Tween 80 produces less methane without affecting VFA production *in vitro* (Kim et al., 2005). However, the improved enzymatic hydrolysis of forage crops depends on the concentration and type of surfactant. Thus, little is understood about the mechanism whereby Tween 80 enhances cellulase activity in the rumen. Since cellulases are composed of several functional modules including catalytic and cellulose binding domains (Bayer et al., 1998), the mechanism through which Tween 80 enhances activity may be clarified by studying its influence on these functional regions.

Several cellulolytic enzymes of *Trichoderma sp.* are extensively used in the degradation of crystalline cellulose (Van Tilbeurgh et al., 1986; Tomme et al., 1988; Lemos et al., 2003). The activity of the commercial enzyme complex, Meicelase (P-1, Meiji Seika, Tokyo, Japan) is attributable not only to several endoglucanases and cellobiohydrolases (Srisodsuk, 1994; Saloheimo et al., 1997), but also to a cellulose binding module linked through glycosylate to the catalytic module. We examined the relationships among surfactant, cellulolytic enzyme and fibrolytic substrate be determining the extent to which whole CBH I of Meicelase and its catalytic module adsorb onto ball-milled cellulose (BMC). Furthermore, the extent to which Tween 80 enhanced the enzymatic hydrolysis of BMC was assessed by measuring the release of reducing sugars. Scanning electron microscopy (SEM) determined whether Tween 80 caused morphological changes in BMC.

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## MATERIALS AND METHODS

### Purification of CBH I and experimental treatments

Either whole CBH I (native CBH I) or its catalytic module (core CBH I) were mixed with the following concentrations of Tween 80: native CBH I alone (C0), and with 1% (C1) or 2% (C2) Tween 80; core CBH I alone (P0), and with 1% (P1) or 2% (P2) Tween 80. All ratios of Tween 80 were mixed on a w/w (substrate) basis and the concentrations of native and core CBH I were adjusted to 1% of the weight of BMC dissolved in 0.1 M potassium phosphate buffer (pH 7.0).

Native CBH I was purified from Meicelase P-1 (a cellulase derived from *Trichoderma viride*; Meiji Seika, Tokyo, Japan) by anion exchange chromatography with a HiTrap Q column (1 ml, Amersham Pharmacia Biotech AB,

**Table 1.** Effect of Tween 80 on concentration of enzyme proteins in supernatant

| Time (h) | Tween 80 (%, w/w) | | | Significant effect | | |
|---|---|---|---|---|---|---|
| Enzyme (En) | 0 | 1 | 2 | En | Tw | En×Tw |
| 10 min (μg/ml) | | | | p<0.001 | p<0.001 | p<0.141 |
| C | 13.83±5.42$^{e}$ | 52.58±2.50$^{c}$ | 69.67±2.92$^{b}$ | | | |
| P | 40.04±0.83$^{d}$ | 70.08±4.17$^{b}$ | 88.42±5.00$^{a}$ | | | |
| 1 h (μg/ml) | | | | p<0.001 | p<0.001 | p<0.002 |
| C | 14.20±1.05$^{e}$ | 51.54±1.16$^{c}$ | 70.45±0.87$^{b}$ | | | |
| P | 40.26±1.06$^{d}$ | 70.33±0.98$^{b}$ | 90.36±2.72$^{a}$ | | | |
| 2 h (μg/ml) | | | | p<0.001 | p<0.001 | p<0.015 |
| C | 14.12±0.57$^{e}$ | 51.69±2.02$^{c}$ | 69.67±1.24$^{b}$ | | | |
| P | 41.63±3.65$^{d}$ | 71.75±2.09$^{b}$ | 90.73±0.52$^{a}$ | | | |
| 3 h (μg/ml) | | | | p<0.001 | p<0.001 | p<0.007 |
| C | 14.24±1.01$^{e}$ | 51.33±1.76$^{c}$ | 71.87±2.26$^{b}$ | | | |
| P | 39.20±2.31$^{d}$ | 69.86±0.57$^{b}$ | 89.39±2.06$^{a}$ | | | |
| 4 h (μg/ml) | | | | p<0.001 | p<0.001 | p<0.216 |
| C | 15.89±1.31$^{e}$ | 50.03±2.19$^{c}$ | 69.06±1.16$^{b}$ | | | |
| P | 42.12±3.00$^{d}$ | 70.80±1.56$^{b}$ | 92.67±4.36$^{a}$ | | | |
| 5 h (μg/ml) | | | | p<0.001 | p<0.001 | p<0.198 |
| C | 14.90±0.81$^{e}$ | 50.05±0.58$^{c}$ | 69.79±2.56$^{b}$ | | | |
| P | 39.16±2.09$^{d}$ | 70.31±2.75$^{b}$ | 90.57±1.83$^{a}$ | | | |

$^{a, b, c, d}$ Significant differences by Tukey's multiple test.
Values are means of 3 replicates.

Sweden, Uppsala) using a linear gradient (Bhikhabhai et al., 1984). The purification was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and enzymatic activity on carboxymethyl cellulase was measured by photometry.

The catalytic module of CBH I was obtained from the protease, papain (Wako Pure Chemicals, Tokyo, Japan), which hydrolyzes the linkage of the catalytic and cellulose-binding modules (Van Tilbeurgh et al., 1986). Core CBH I was isolated by anion exchange chromatography. Proteolysis was confirmed by 12% SDS-PAGE, using molecular mass markers (LMW Calibration, Amersham Biosciences, New Jersey, USA) as standards.

Cellulose (BMC, W-300G, Nippon paper Chemicals Co. Ltd., Tokyo, Japan) was prepared by grinding in a ceramic ball mill (Takano et al., 1992). The surfactant Tween 80 (polyoxyethylene sorbitan monooleate) was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

**Measurements of adsorbed CBH I onto substrate and enzymatic activity**

The adsorption of native and core CBH I onto ball-milled cellulose (BMC) was estimated as the protein concentration in the supernatant after mixing for 10 min and 1, 2, 3, 4 and 5 h with various ratios of Tween 80, because a preliminary investigation showed that cellulolytic activity declined after 4 h at room temperature and centrifugation at 7,000 rpm for 2 min. The protein concentration in the supernatant was determined by the Folin-Lowry reaction using BSA as the standard (Lowry et al., 1951).

Cellulase activity was assayed after 3% (w/v) BMC dissolved in the buffer described above, was incubated at 38°C for 1, 2, 3, 4 and 5 h without shaking. Samples were centrifuged at 7,000 rpm for 5 min, and reducing sugars in the supernatant were determined using dinitrosalicylic acid (Miller, 1959)

**SEM observation of filter paper treated with native and core CBH I**

Samples of filter paper (No.1, Whatman International Ltd, Maidstone, UK) were soaked in C0, C1, P0 and P1 at 38°C for 10 min, fixed in 2.5% (v/v) glutaraldehyde for 2 h and then washed with 0.1 M sodium phosphate buffer (pH 7.2) for 30 min three times at 30 min intervals. The filters were dehydrated through a graded ethanol series, dried by critical-point drying with liquid $CO_2$, and sputtered with gold (Bosworth et al., 2001). Samples were observed using SEM (Hitachi S-4000, Tokyo, Japan).

**Statistical analysis**

Data were analyzed and mean differences were determined using Tukey's multiple range test according to the SAS (Statistical Analysis Systems Institute Inc. 1989) procedure.

## RESULTS AND DISCUSSION

The molecular mass of the purified native and core CBH I proteins were 65 and 56 kDa, respectively (Figure 1). The amounts of native and core CBH I in the supernatant

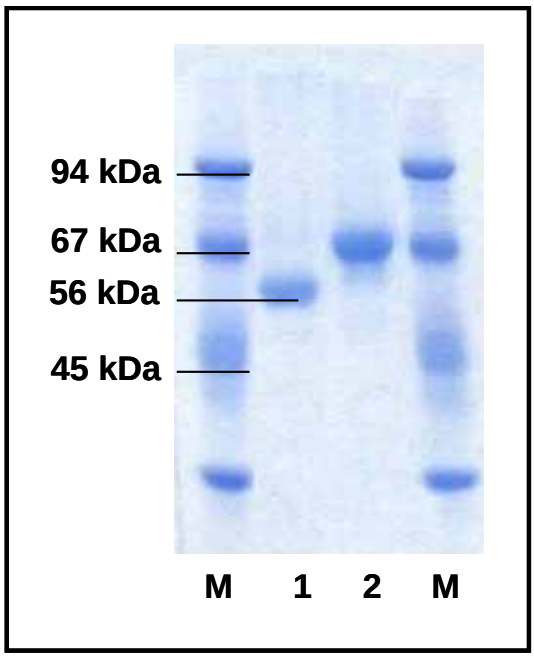


**Figure 1.** Confirmation of limited proteolysis of CBH I by papain. M, Marker; 1, Core CBH I (56 kDa); 2, native CBH I (65 kDa).

increased ($p<0.001$) with increasing concentrations of Tween 80 (Table 1). This finding indicated that Tween 80 decreased the amounts of the native and core CBH I that adsorbed onto BMC, findings that were in agreement with the results of studies of non-ionic surfactants (Park et al., 1991; Helle et al., 1993; Eriksson et al., 2003). The concentrations of native CBH I at 3 concentrations of Tween 80 were also lower than the corresponding concentrations of core CBH I. The adsorption of native or core CBH I onto BMC was not affected by the incubation period, suggesting that the enzymatic reaction was generally maintained.

The amount of reducing sugars released from BMC by native CBH I was increased ($p<0.001$) by Tween 80 (Figure 2). Especially at 1 h of incubation, the amounts considerably differed among Tween 80 concentrations, although the differences were reduced with increasing incubation time. In contrast, the amount of reducing sugars released by core CHB I remained low, suggesting little response to Tween 80 for any length of time.

The surface of untreated filter paper was smooth and sleek (Figure 3a), whereas that after exposure to Tween 80 was swollen and flaky (Figure 3b). The surface exposed to native CBH I was remarkably disordered and peeled (Figure 3c), whereas core CBH I caused some cracks and filaments (Figure 3d). After immersion in native CBH I and Tween 80, the filter paper surface was obviously disordered and eroded (Figure 3e). In contrast, core CBH I and Tween 80 caused limited morphological changes with some cracks, flakes and filaments (Figure 3f).

The surfactant Tween 80 considerably improved Meicelase hydrolysis, which was in agreement with the findings of others (Fendler and Fendler, 1975; Castanon and Wilke, 1981; Kim et al., 1982; Ooshima et al., 1986; Helle

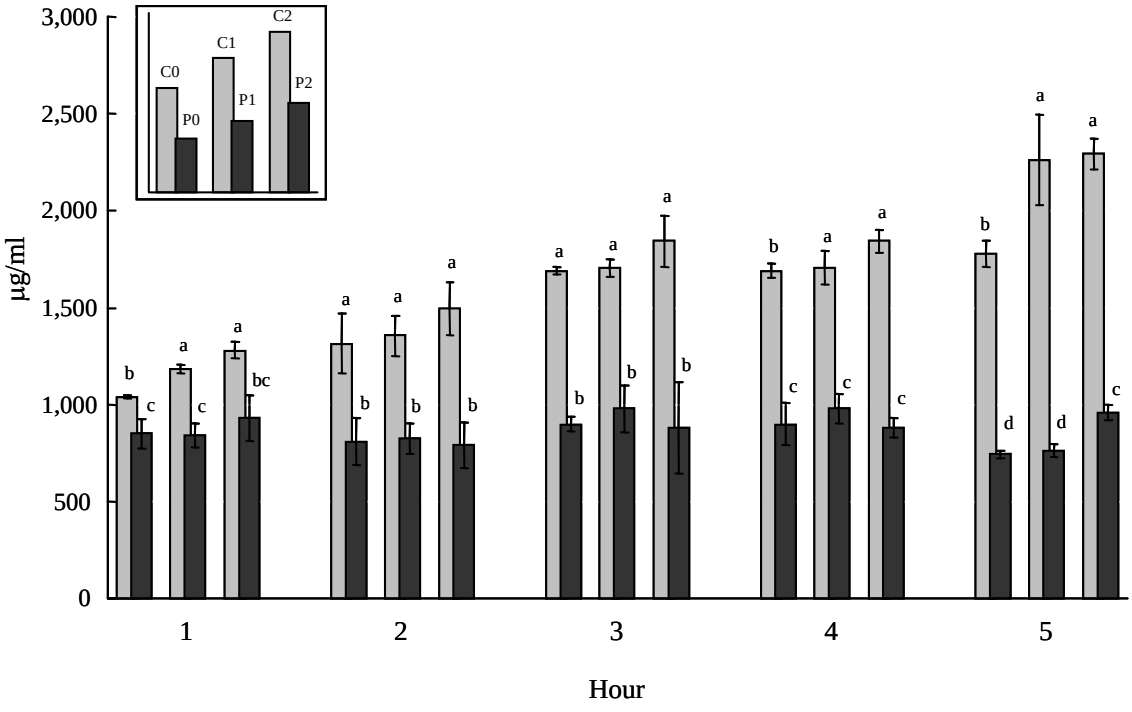


**Figure 2.** Concentration of reducing sugars in supernatant after reaction with ball-milled cellulose (BMC). C0, native CBH I; C1, native CBH I and 1% Tween 80; C2, native CBH I and 2% Tween 80; P0, core CBH I; P1, core CBH I and 1% Tween 80; P2, core CBH I and 2% Tween 80. All ratios (%) are w/w substrate. [a, b, c, d] Means with superscripts significantly differ ($p<0.05$).

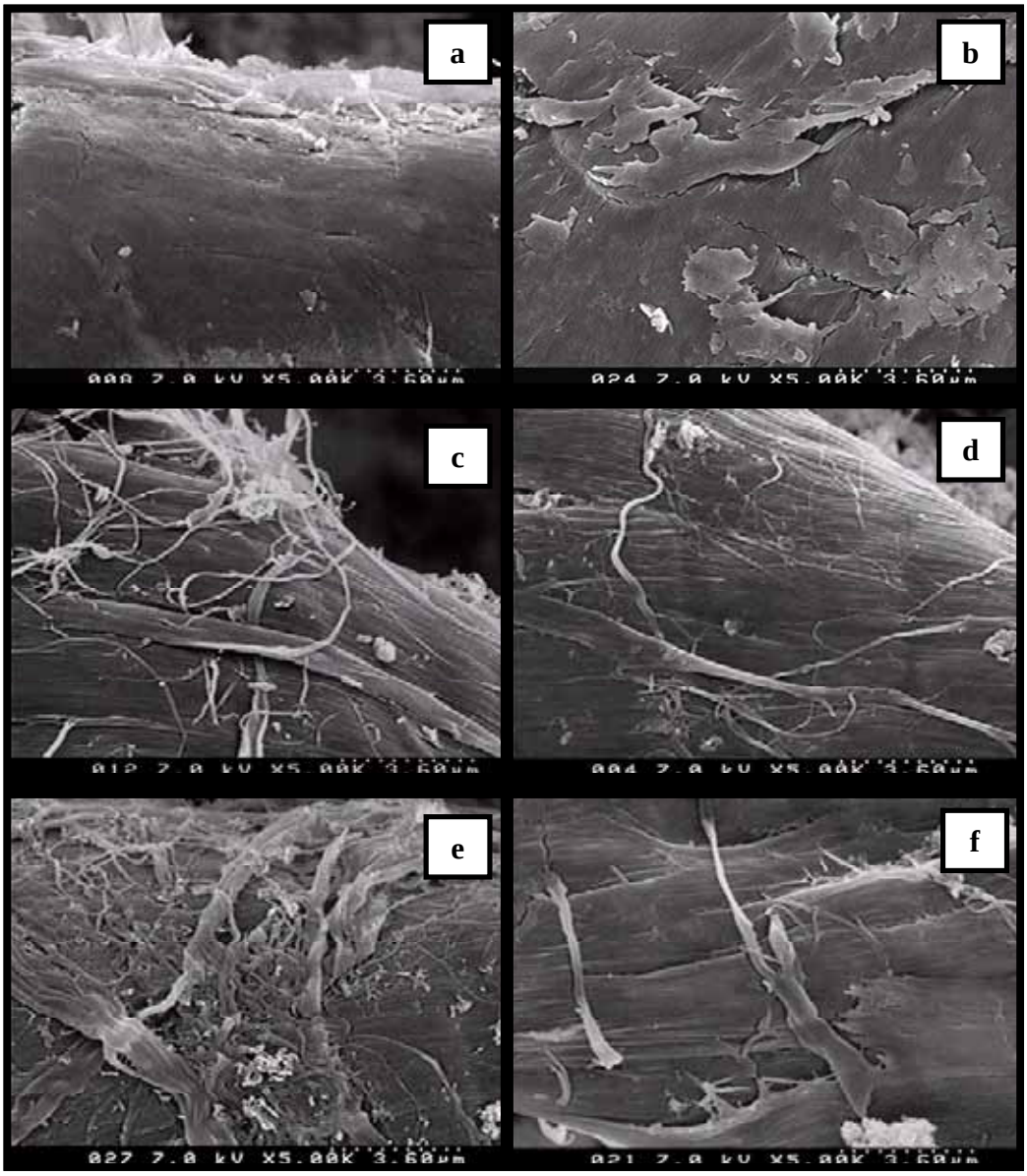


**Figure 3.** SEM observation of surface state of filter paper. a) No-Tween 80, b) Tween 80, c) native CBH I, d) core CBH I, e) native CBH I and Tween 80, f) core CBH I and Tween 80.

at al., 1993; Eriksson et al., 2002; Goto et al., 2003a, 2003b). We also found that Tween 80 increased the ability of CBH I with, but not without its cellulose-binding module to enzymatically hydrolyze cellulose. This finding suggests that Tween 80 is closely associated with interaction between the cellulose-binding module and substrate, as indicated by the consistently higher concentrations of core CBH I proteins in the supernatant compared with those of native CBH I. These findings were also consistent with those of Tomme et al. (1988), who showed that the amount of reducing sugars released by core CBH I isolated from *Trichoderma reesei* was decreased to one-seventh of that released by native CBH I. However, within the enzymatic activity of native CBH I, the concentration of the reducing sugars was higher in the presence, than in the absence of Tween 80, suggesting that the responses of native and core CBH I are antagonistic. Therefore, the enzyme-substrate interaction improved by surfactant Tween 80 would be associated with reduced inactivation of the enzyme bound to substrate and increased mobilization of the enzyme among substrate reaction sites (Castanon and Wilke, 1981; Helle et al., 1993; Kaar and Holzapple, 1998; Eriksson et al., 2002). In addition, reduced thermal denaturation or denaturation by shear forces increase enzymatic stability and allow the enzyme to remain active for a longer period (Kim et al., 1982; Kaar and Holzapple, 1998).

The response of native CBH I to the surfactant concentration was inconsistent with the response of enzyme

binding to BMC at various Tween 80 concentrations. This finding was in agreement with those of Goto et al. (2003b), who showed that the degradability of dry matter from leaf blades of young orchard grass was increased at Tween 80 concentrations over 0.01%, but that the enzyme binding capacity was increased only at 0.2% Tween 80. In addition, our SEM observations of filter paper soaked in Tween 80 showed changes in the morphological structure of the surface. Thus, surfactants can initially alter cellulose ultrastructures to increase vulnerability to enzymatic attack (Helle et al., 1993). Enzyme proteins might bind to hydrophobic regions of cellulose after a surfactant has increased its wettability (Goto et al., 2003b).

In conclusion, the results of the present study suggest that the improvement of enzymatic hydrolysis by surfactants depends upon synergism between enzyme stability and substrate accessibility. However, further research is needed to elucidate the effect of surfactants on the rate and extent of enzyme turnover.

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