J Wood Sci (2001) 47:135–140

ORIGINAL ARTICLE

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Inhibition of *Trichoderma* cellulase activity by a stilbene glucoside from *Picea glehnii* bark

Received: March 22, 2000 / Accepted: May 22, 2000

Abstract The stilbene glucoside isorhapontin (5,4′-dihydroxy-3′-methoxystilbene-3-β-D-glucoside) is the major constituent of the ethyl acetate extracts from *Picea glehnii* bark. Isorhapontin inhibited the hydrolytic activity of *Trichoderma* cellobiohydrolase I (CBH I) for both bacterial microcrystalline cellulose and the soluble cello-oligosaccharide celloheptaitol. The inhibitory effect for celloheptaitol, however, was more drastic than that for bacterial microcrystalline cellulose. The hydrolytic activity of the CBH I core domain for celloheptaitol was also inhibited by isorhapontin to a similar extent, suggesting that the interaction between isorhapontin and the core domain of CBH I is the reason for this phenomenon. The inhibition of CBH I activity by isorhapontin showed mixed noncompetitive and uncompetitive types in a concentration of the inhibitor of less than 125 μM. The K_i and K_i' values were estimated to be 57.2 and 33.3 μM, respectively. Whereas isorhapontin strongly inhibited CBH I activity, its aglycone isorhapontigenin (3′-methoxy-3,5,4′-trihydroxystilbene) showed almost no inhibition. Consequently, both the stilbenic and the β-glucosidic structures in isorhapontin are essential for the inhibitory effect on CBH I activity. Isorhapontin also inhibited the activity of *Trichoderma* endoglucanase I for celloheptaitol, whereas almost no effect was observed for the activities of both endoglucanases II and III.

Key words Stilbene glucoside · *Picea glehnii* · Cellulase · Inhibitor · *Trichoderma*

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An outline of this work was presented at the 49th Annual Meeting of the Japan Wood Research Society, Tokyo, April 1999

Introduction

Coniferous barks are good sources of phenolic extractives.[1,2] In many coniferous species the content of phenolic extractives exceeds 10% of the dry weight of bark. Monomeric flavanols and polyflavanols, well known as proanthocyanindins or condensed tannins, are present as major components of phenolic extractives in the bark of most coniferous species.[3] A high concentration of stilbene glucosides also exists in the bark of *Picea* species.[4–6]

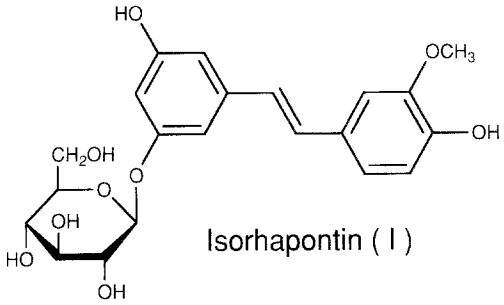
These constituents are presumed to provide resistance against attacks by animals, insects, and microorganisms. In fact, antimicrobial activities of polyflavanols have been widely known,[7,8] and antifungal activities of stilbenes have been reported.[9–12]

In a previous report[13] we described growth inhibition of the wood-staining fungus *Trichoderma viride* by the stilbene glucoside isorhapontin (5,4′-dihydroxy-3′-methoxystilbene-3β-D-glucoside). This fungus has been known as a strong cellulose degrader. In the present work, the effects of isorhapontin on the activities of *Trichoderma* cellulases were investigated.

Materials and methods

Extraction and purification of isorhapontin

Bark of *Picea glehnii* was obtained from freshly felled thinning trees in the Tokyo University Forest, Hokkaido. After air-drying, the outer surface of the bark was removed by a knife. The remaining inner bark was ground to meal by passing it through a 1-mm sieve in a Wiley mill. Then 50 g of the bark meal was dewaxed with *n*-hexane, followed by two extractions with 500 ml of ethyl acetate at room temperature for 24 h. The ethyl acetate extracts were filtered, and the solvent was evaporated in vacuo on a rotary evaporator to weigh the solid. The ethyl acetate extract (3.0 g) was applied to a Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) column (25 × 300 mm) and



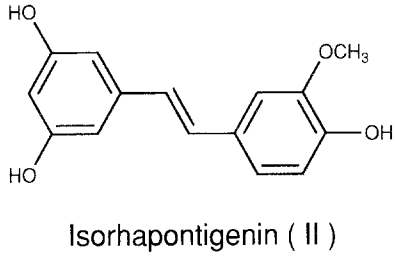


Fig. 1. Chemical structures of isorhapontin (**I**) and isorhapontigenin (**II**)

eluted with ethanol. The fraction containing the major component was further chromatographed on a C18 gel (GL Sciences, Tokyo, Japan) column (30 × 190 mm) with acetonitrile/H_2O 13:87 (v/v). The major component after purification gave a single peak at 18.3 min on high-performance liquid chromatography (HPLC) analysis under the following condition: Cosmosil 5C18-AR column (4.6 × 150 mm, Nacalai Tesque, Kyoto, Japan); solvent system: H_2O followed by a linear gradient up to 50% acetonitrile in 30 ml; flow rate 1.0 ml/min; detection 280 nm. The chemical structure was analyzed by both negative fast atom bombardment-mass spectrometry (FAB-MS) and ^{13}C nuclear magnetic resonance (NMR).

Negative FAB-MS (m/z): 419 ($M-H^-$). ^{13}C-NMR (ppm): signals of the stilbenic residue; 140.3 (C-1), 106.5 (C-2), 160.3 (C-3), 102.2 (C-4), 159.4 (C-5), 108.2 (C-6), 130.4 (C-1′), 110.2 (C-2′), 147.7 (C-3′), 148.6 (C-4′), 115.9 (C-5′), 121.3 (C-6′), 126.7 (Cα), 130.0 (Cβ), 56.2 (—OCH_3); signals of the glucosidic residue; 103.8 (C-1), 74.7 (C-2), 77.8 (C-3), 71.4 (C-4), 78.0 (C-5), 62.7 (C-6). By comparison with the data in an earlier report[14] the compound was identified as the stilbene glucoside isorhapontin (5,4′-dihydroxy-3′-methoxystilbene-3-β-glucoside), shown as **I** in Fig. 1.

Preparation of isorhapontigenin

Isorhapontin (1.0 g) was incubated with 500 U almond β-glucosidase (Wako Pure Chemical Industries, Osaka, Japan) in 100 ml of 100 mM sodium acetate, pH 5.0, at 30°C for 6 h. The mixture was extracted three times with 100 ml ethyl acetate. The solvent was evaporated in vacuo on a rotary evaporator. The resultant solid was dissolved in an aliquot of 50% acetonitrile and then chromatographed on the C18 gel column previously equilibrated with acetonitrile/H_2O 27:73 (v/v). The reaction product gave a peak at 22.7 min of retention time on HPLC analysis. The chemical structure of the purified product was analyzed.

Negative FAB-MS (m/z): 257 ($M-H^-$). ^{13}C-NMR (ppm): 140.9 (C-1), 105.7 (C-2), 159.6 (C-3), 102.7 (C-4), 159.6 (C-5), 105.7 (C-6), 130.4 (C-1′), 110.1 (C-2′), 147.5 (C-3′), 148.6 (C-4′), 115.9 (C-5′), 121.2 (C-6′), 127.0 (Cα), 129.4 (Cβ), 56.2 (—OCH_3). From these data, the product was identified as isorhapontigenin (3′-methoxy-3,5,4′-trihydroxystilbene), shown as **II** in Fig. 1.

Preparation of enzymes

Meicelase, a *Trichoderma viride* cellulase mixture, was kindly provided from Meiji Seika Co. (Tokyo, Japan). Cellobiohydrolase I (CBH I), the major component of the *Trichoderma* cellulase system, was purified from Meicelase as previously described.[15] The core domain of CBH I was obtained by partial proteolysis of the intact enzyme according to the method of Van Tilbeurgh et al.[16] CBH I (0.39 μmol) was treated with 1.3 U papain (Elastin Products, Owensville, MO, USA) in 5 ml 20 mM phosphate buffer containing 100 μM dithiothreitol, pH 7.0, at 30°C for 120 min. After the treatment, the reaction mixture was applied to a DEAE-Toyopearl 650 S (Tosoh, Tokyo, Japan) column (22 × 400 mm) equilibrated with 20 mM phosphate buffer, pH 7.0, and eluted with a linear gradient of KCl from 0 to 250 mM in 1500 ml of the same buffer. The active fraction was collected, and the buffer solution was changed to 20 mM phosphate buffer containing 1 M ammonium sulfate, pH 7.0. Further purification of the core domain was performed on a Phenyl-Toyopearl 650 M (Tosoh) column (16 × 120 mm) equilibrated with the same buffer. The core domain was eluted from the column by the same buffer, whereas the intact CBH I remained on the gel under this condition.

The purified samples of three endoglucanases – endoglucanase I (EG I), endoglucanase II (EG II), and endoglucanase III (EG III) – from *Trichoderma reesei*, was a kind gift from Dr. Tim Fowler, Genencor International (Palo Alto, CA, USA).

Cellobiose dehydrogenase (CDH) was produced in the culture solution of *Phanerochaete chrysosporium* grown on cellulose and purified as described in our previous paper.[17,18]

Cellulase assays

Two methods were applied for the cellulase assay depending on its purpose. Bacterial microcrystalline cellulose (BMCC) and celloheptaitol were prepared according to previous reports and were used as substrates.[15,19]

Method 1. To investigate the inhibiting ability of isorhapontin on the hydrolysis of BMCC and celloheptaitol by CBH I, the activity was monitored by the potentiometric method based on the CDH-ferricyanide redox system as previously described.[15,20] The cellulose sample (0.05% w/v)

was incubated with 0.6μM CDH, 500μM ferricyanide, 500μM ferrocyanide in 100mM sodium acetate, pH 5.0, at 30°C. To start the reaction, 1.4μM CBH I or its core domain was added. The hydrolytic rate of cellulose substrate was continuously monitored in the presence or absence of 1.0mM isorhapontin by following the changes in electrode potential. Prior to this assay, it had been confirmed that isorhapontin has no effect on CDH activity in the working condition.

Method 2. To investigate the kinetics of CBH I in the presence of the stilbene glucoside isorhapontin or its aglycone isorhapontigenin, activity was detected by the photometric method based on the CDH–cytochrome c redox system. That is, 50–300μM celloheptaitol was incubated with 50μM cytochrome c, 62nM CDH, 0–500μM isorhapontin in 100mM sodium acetate, pH 5.0. The enzymatic reaction was started by adding of 137nM CBH I. The amount of reducing sugar released was measured by the increase of absorption at 550nm due to the reduction of cytochrome c ($\Delta\varepsilon_{550}$ = 15.0mM^{-1}cm^{-1}) as previously described.[21] Inhibition constants, K_i and K_i', of isorhapontin for CBH I activity were calculated from the Lineweaver-Burk plots using the Delta Graph 4.0 (Deltapoint). The inhibitory effect of 250μM isorhapontigenin on CBH I activity was assayed under the same conditions. This method was also applied for measuring the effects of 250μM isorhapontin on the activity of *Trichoderma* endoglucanases.

Results

Cellobiohydrolase I (CBH I) is known to be the major component of the *Trichoderma* cellulase system.[22] Thus, CBH I was chosen as the representative cellulase for investigation. First, the inhibitory effect of the stilbene glucoside isorhapontin (**I**) on the hydrolytic activity of CBH I for bacterial microcrystalline cellulose (BMCC) and celloheptaitol was examined. As shown in Fig. 2, the hydrolytic activity of CBH I for both substrates was obviously inhibited in the presence of 1mM isorhapontin. However, inhibition of CBH I activity for celloheptaitol was more significant than that for BMCC, suggesting that the isorhapontin interacts with the core domain of CBH I. To confirm this point, the effect of isorhapontin on the hydrolytic activity of the core domain, which had been prepared by partial proteolysis of the intact CBH I, was examined. As shown in Fig. 3, the inhibitory effect of isorhapontin on the hydrolysis of celloheptaitol was preserved in the core domain to the same extent as the intact CBH I.

The kinetics of CBH I activity for the soluble cello-oligosaccharide celloheptaitol was investigated in the presence of isorhapontin. As shown in Fig. 4A, CBH I activity for celloheptaitol was inhibited in correlation with the amounts of isorhapontin. From the Lineweaver-Burk plots (Fig. 4B), the effect of isorhapontin on CBH I activity was presumed to be a mixed manner of noncompetitive and uncompetitive inhibition in a concentration range of the inhibitor of less than 125μM. The K_i and K_i' values calculated for this range were 57.2 and 33.3μM, respectively. However, at an isorhapontin concentration of more than 250μM, additional factors might be involved in the inhibiting effects on CBH I activity.

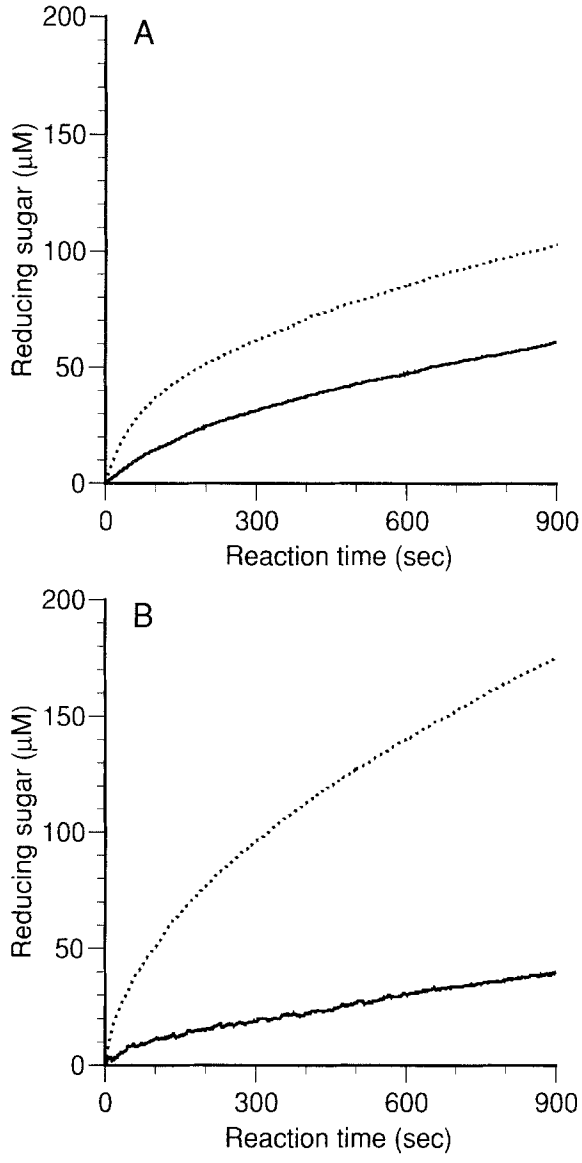


Fig. 2. Effect of the stilbene glucoside isorhapontin on the hydrolytic activity of cellobiohydrolase I (CBH I) for bacterial microcrystalline cellulose (BMCC) (**A**) and celloheptaitol (**B**). Amount of reducing sugar released was determined by the potentiometric assay according to method 1 under Materials and methods. *Solid line*, 1mM isorhapontin; *dotted line*, control

To investigate the function of the glucosidic residue in isorhapontin, the inhibitory effect of isorhapontin and its aglycone isorhapontigenin (**II**) on CBH I activity for celloheptaitol was compared. As shown in Fig. 5, the relative activity of CBH I decreased significantly in the presence of 250μM isorhapontin, whereas the activity was only slightly affected at the same concentration of isorhapontigenin. Consequently, both the stilbenic and the β-glucosidic structures in isorhapontin seemed to be essential for the inhibitory action of CBH I activity.

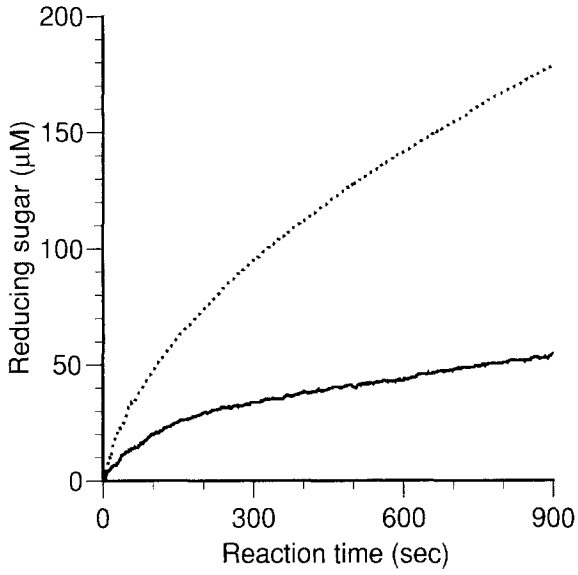


Fig. 3. Effect of the stilbene glucoside isorhapontin on the hydrolytic activity of the CBH I core domain for celloheptaitol. Experimental conditions were as for method 1. *Solid line*, 1 mM isorhapontin; *dotted line*, control (without addition of isorhapontin)

The effects of isorhapontin on the hydrolytic activities of *Trichoderma* endoglucanases for celloheptaitol were also examined. As shown in Fig. 6, only the hydrolytic activity of EG I for celloheptaitol was inhibited in the presence of isorhapontin well as CBH I, whereas almost no effect was observed for the activities of EG II and EG III.

Discussion

Chemical analysis demonstrated that the major part of the extracts from *P. glehnii* bark was composed of the stilbene glucoside isorhapontin (**I**), and the yield of this compound was estimated to be 9.6% (w/w) of the dried bark meal on HPLC analysis (data not shown). The wide distribution of stilbene glucosides in the barks of *Picea* species are known,[4–6] and antifungal activities of stilbenes have been reported.[9–12] However, as far as we know, this is the first report to demonstrate the inhibition of cellulase activity by stilbenes. Although the inhibition of cellulase activity by polyflavanols has been reported,[23] the ability of isorhapontin to inhibit CBH I activity is much stronger than that of polyflavanols.

Isorhapontin inhibited the hydrolytic activity of *Trichoderma* CBH I for both the insoluble BMCC and the soluble celloheptaitol. However, the inhibitory effect of isorhapontin on CBH I activity for celloheptaitol were more significant than that for BMCC. CBH I is composed of two domain structures: the large-core domain with a catalytic function for hydrolysis and the small cellulose binding domain.[24] It has been reported that combining the functions of the core domain and the cellulose-binding domain are essential for hydrolyzing insoluble crystalline celluloses, whereas only the core domain is necessary for hydrolyzing

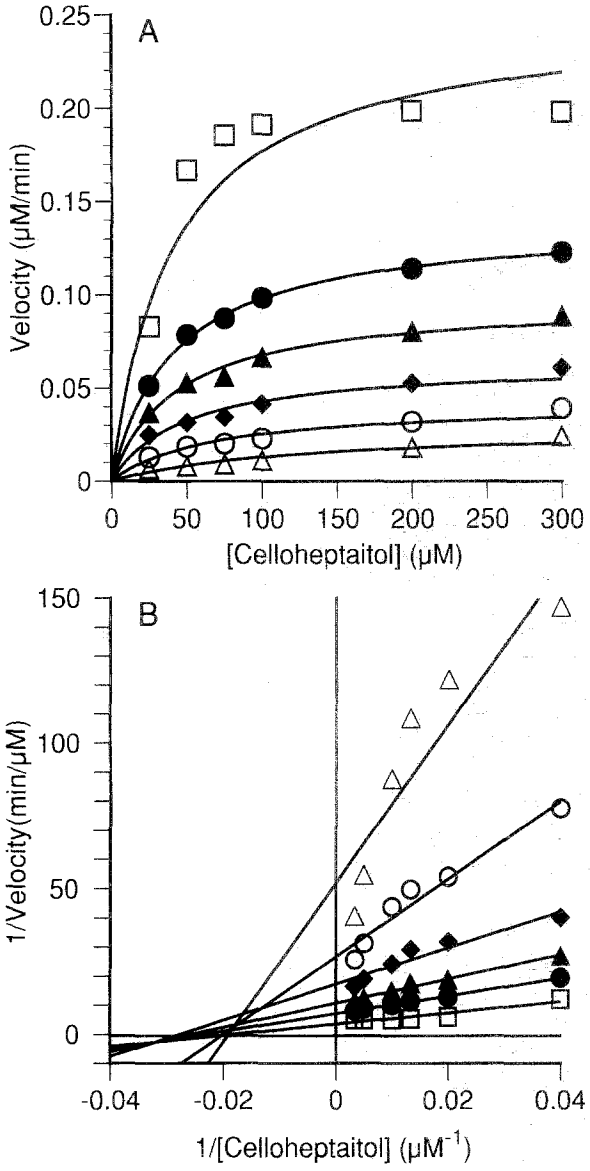


Fig. 4. Hydrolytic rate of celloheptaitol by CBH I in the presence of various amounts of the stilbene glucoside isorhapontin (**A**) and their Lineweaver-Burk plots (**B**). Amount of reducing sugar released was determined by the photometric assay according to method 2 in Materials and Methods. Concentration of isorhapontin: *open triangles*, 500 μM; *open circles*, 250 μM; *diamonds*, 125 μM; *filled triangles*, 62.5 μM; *filled circles*, 31.3 μM; *squares*, control (without addition of isorhapontin)

soluble cellulosic substrates.[16,25] Therefore, the significant inhibition of CBH I activity for celloheptaitol seems to be an interaction between isorhapontin and the core domain. This idea was proved by the experimental result that the hydrolysis of celloheptaitol by the core domain was inhibited by isorhapontin to a similar extent.

Inhibition constants of isorhapontin for CBH I activity are similar to that of cellobiose, for which K_i is 53 μM for CBH I activity (data not shown), indicating that the inhibitory effect of isorhapontin is strong enough to presume the site-specific binding of it in the core domain of CBH I. However, the effect of isorhapontin on CBH I activity

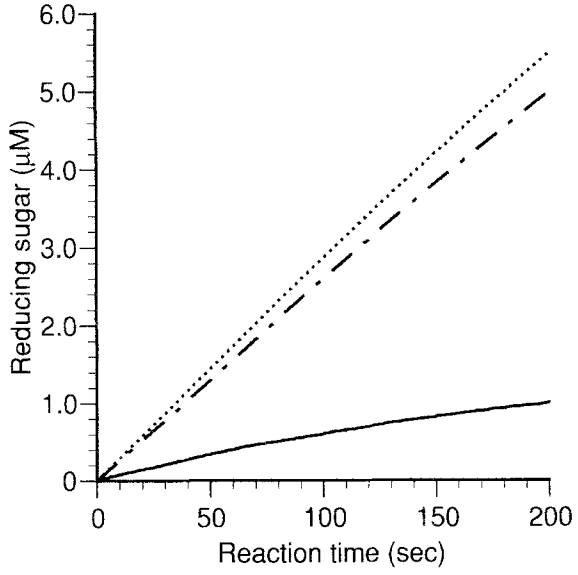


Fig. 5. Hydrolytic activity of CBH I for celloheptaitol in the presence of the stilbene glucoside isorhapontin or its aglycone isorhapontigenin. Experimental condition was as in method 2. *Solid line*, 250μM isorhapontin; *dot-dashed line*, 250μM isorhapontigenin; *dotted line*, control (without addition of isorhapontin)

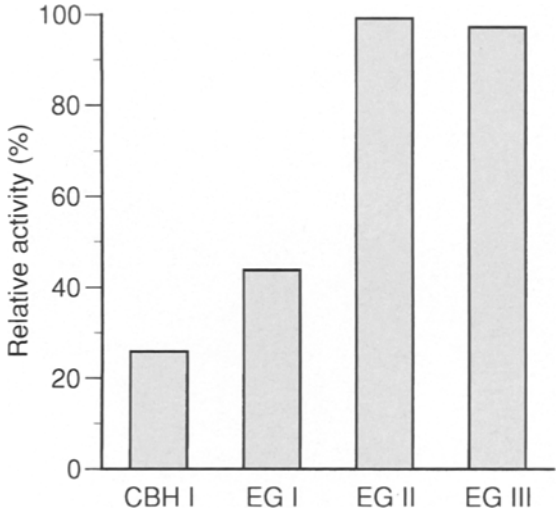


Fig. 6. Relative activity of *Trichoderma* cellulases on the hydrolysis of celloheptaitol in the presence of 250μM isorhapontin compared with control (without addition of isorhapontin). Experimental conditions were as in method 2. *EG*, endoglucanase

shows mixed noncompetitive and uncompetitive inhibition, suggesting that the interaction mechanism between isorhapontin and the core domain of CBH I is complicated. The active site on the core domain is a tunnel-like shape of 50 Å length in which nine glucose residues of the cellulose chain can be placed.[26] At the tunnel-like active site, four tryptophan residues are located along the orientation of the cellulose chain.[27] Therefore, a possible explanation for the inhibition is a hydrophobic interaction between the stilbenic residue in isorhapontin and one of the tryptophan residues at the active site of CBH I. Although the stilbene glucoside isorhapontin significantly inhibits CBH I activity, the effect of its aglycone isorhapontigenin is much less. This suggests that the stilbenic and β-glucosidic structures must be combined for the inhibitory action of isorhapontin at the active site of CBH I. Furthermore, three-dimensional analysis of the CBH I–isorhapontin complex is required to determine details of the inhibition mechanism.

Among three *Trichoderma* endoglucanases examined, only the activity of EG I was significantly inhibited by isorhapontin. According to the recent classification of *Trichoderma* cellulases, depending on the structural analysis of glycosyl hydrolases, EG I belongs to the GH-family 7, as like as CBH I.[28] Thus, the inhibitory effects of isorhapontin are presumed to be due to an interaction to a preserved structure of the core domain in the GH-family 7 cellulases.

Acknowledgment The authors thank Syohei Sakuda and Ichio Furihata for their technical support on the MS and NMR measurements, respectively.

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