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A Novel Glucosylation Enzyme: Molecular Cloning, Expression, and Characterization of *Trichoderma viride* JCM22452 α-Amylase and Enzymatic Synthesis of Some Flavonoid Monoglucosides and Oligoglucosides

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It was found that commercial cellulase preparations from *Trichoderma viride* showed transglucosylation activity toward (+)-catechin and (−)-epigallocatechin gallate (EGCG) using dextrin as a glucosyl donor. To isolate the enzyme exhibiting transglucosylation activity toward (+)-catechin and EGCG, the present study isolated the cDNA encoding the *T. viride* JCM22452 α-amylase homologue (TRa2), which showed high amino acid sequence identity to functionally uncharacterized α-amylase homologues from other ascomycetes, which also produced some (+)-catechin and EGCG glucosides. TRa2 was able to glucosylate a wide range of natural flavonoids, particularly (+)-catechin and EGCG, and to hydrolyze maltooligosaccharides (k_{cat}/K_m for maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were 1.98, 45.2, 58.3, 97.4, and 92.6 s^{-1} mM^{-1}, respectively) except maltose but could not transfer the monoglucosyl residue to maltooligosaccharides. By ^{1}H NMR and ^{13}C NMR, the structures of several novel glucosides obtained by commercial cellulase preparations from *T. viride* and TRa2 were determined as (+)-catechin 5-*O*-α-D-glucopyranoside, (+)-catechin 5-*O*-α-D-maltoside, (+)-catechin 4′-*O*-α-D-maltoside, EGCG 5-*O*-α-D-glucopyranoside, and EGCG 7-*O*-α-D-maltoside. One of these glucosides, EGCG 5-*O*-α-D-glucopyranoside, showed higher heat stability and solubility and lower astringency and astringent stimulation than its aglycon, suggesting that EGCG glucosides are functionally superior to EGCG as food additives.

KEYWORDS: α-Amylase; *Trichoderma viride*; glucosylation; oligoglucoside

INTRODUCTION

Glycosylation is a useful method for the structural and functional modification of bioactive compounds. It enhances their solubility, physicochemical stability, bioactivity, and intestinal absorption and also improves their taste qualities. Enzymatic transglycosylation is mainly catalyzed by glycosyltransferase (GT) and glycoside hydrolase (GH). In their reactions, GHs use a polysaccharide or oligosaccharide as a glycosyl donor, whereas GTs use a nucleotide-activated sugar. Thus, the production of glycosides by GHs is less expensive than by GTs. Recently, it was reported that some GHs, mainly enzymes of the GH family 13 (also called the α-amylase family), such as cyclodextrin glucanotransferase (*1*), α-glucosidase (*2*−*4*), α-amylase (*5*−*7*), and sucrose phosphorylase (*8*−*11*), are able to transfer the monoglucosyl or oligoglucosyl group to the hydroxyl group of alcohols and phenols. The resultant glucosylated natural flavonoids exhibit increased water solubility, enhanced stability against light or oxidation, and improved pharmacological properties compared with the parent flavonoids (*8, 9, 12, 13*). Moreover, it was shown that the intestinal absorption of quercetin oligoglucosides was higher than that of its monoglucoside (*14*). Thus, glycosylation is an important method for the structural modification of bioactive compounds; however, the positions, number, and length of the sugar moieties are also predicted to be significant factors (*15*).

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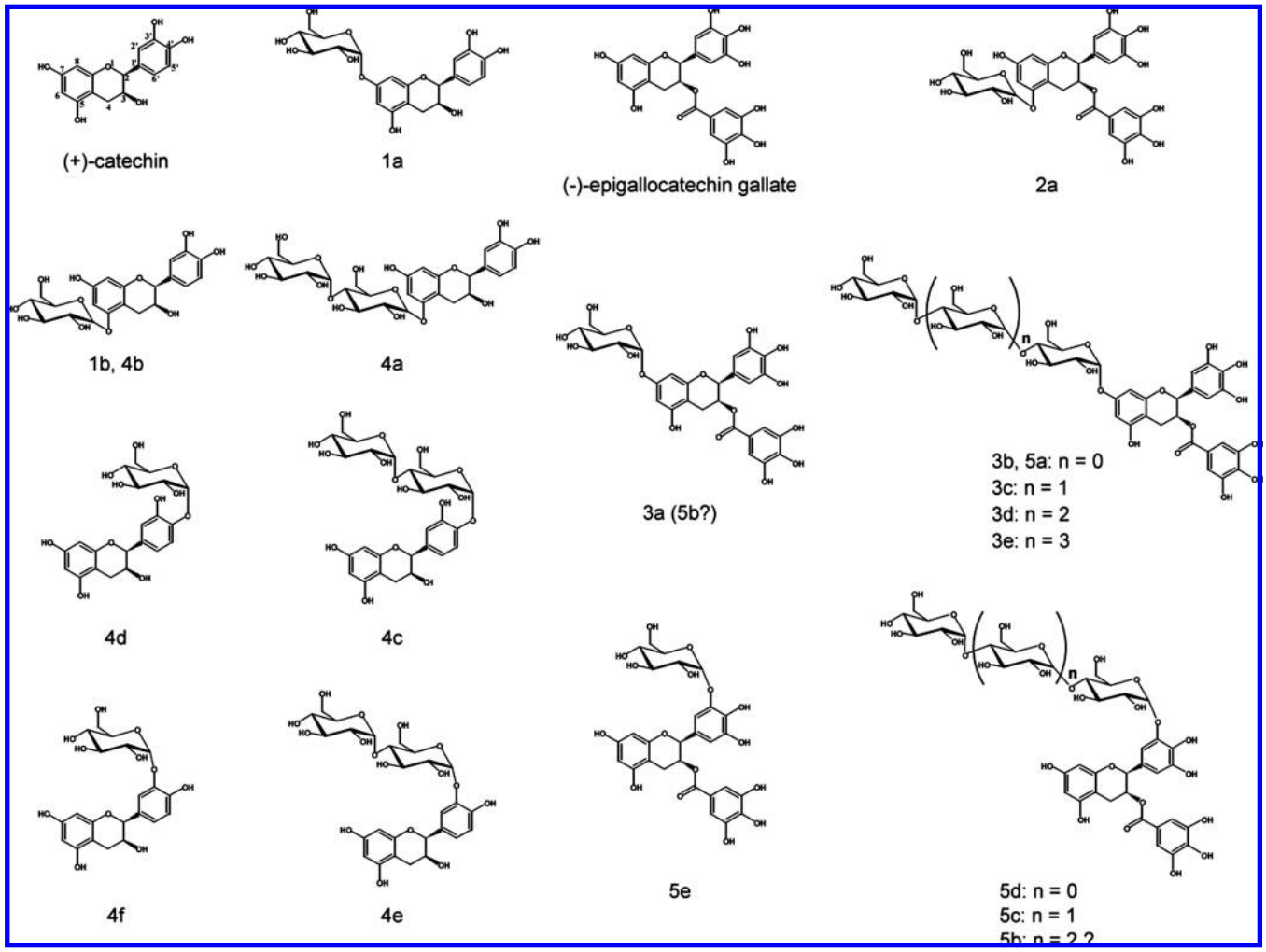

Figure 1. Structures of catechins and the glycosides examined for transglucosylation and produced by commercial enzyme preparations and TRa2, respectively.

Flavonoids are a very large and important group of polyphenolic natural products in vascular plants and Bryophytes. They play various important roles in the growth and development of plants as pigments, antimicrobials, UV protectants, and signal molecules in plant−microbe interaction. Furthermore, flavonoids are beneficial to human health because of their antimicrobial, anticancer, and radical-scavenging properties. Catechin and related flavonoids (catechins, **Figure 1**) contained in green tea are one of the best-known classes of flavonoids. (−)-Epigallocatechin gallate (EGCG, **Figure 1**), the most abundant of the green tea catechins, appears to play a role in the prevention of dental caries and the regulation of plasma lipid levels in addition to the effects described above. However, the use of catechins as food additives is limited because they are poorly soluble in water, are easily degraded in aqueous solution, and have an astringent taste. To overcome these problems, numerous studies have been conducted concerning the glycosylation of catechins. However, the majority of catechin glucosides are monoglucosides or diglucosides, which have one or two glucose moieties in the molecule, respectively, but not oligoglucosides, which have an oligosaccharide in the molecule; this is because the majority of transglucosylations were performed using sucrose as a glucosyl donor (*8, 9, 12, 16−18*). To obtain oligoglucosides, the transglucosylation reaction should be performed using α-amylase or cyclodextrin glucanotransferase as an enzyme and starch or dextrin as a glucosyl donor.

The genus *Trichoderma* comprises a great number of fungal strains that are widely used in industrial applications because of their ability to produce extracellular hydrolases, such as cellulase, hemicellulase, xylanase, and chitinase (*19*). On the other hand, it is known that this genus secretes several starch-acting hydrolases, such as glucoamylase and α-amylase (*20−22*). However, there has been no report of the cloning of such genes and transglucosylation using these enzymes.

It was found that the commercial enzyme preparations from *Trichoderma viride* to be used as cellulase or β-glucosidase also transglucosylate (+)-catechin and (−)-epigallocatechin gallate (EGCG) using dextrin as a glucosyl donor. It was attempted to isolate the cDNA encoding α-amylase from *T. viride* JCM22452. The protein expressed by the obtained cDNA also exhibited a transglucosylation activity toward flavonoids.

This paper describes the transglucosylation reaction of (+)-catechin and EGCG using commercial enzyme preparations, isolation of the cDNA encoding *T. viride* JCM22452 α-amylase (TRa2), expression of the TRa2 protein, and the transglucosylation reaction with the same flavonoids by TRa2. This study also describes improvement in heat stability, solubility, and taste of the obtained (+)-catechin and EGCG glucosides in comparison with their aglycones.

EXPERIMENTAL PROCEDURES

Materials. Cellulase Onozuka RS and Pancelase BR were products from the Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). Cellulase T Amano 4 was a product from Amano Enzyme (Nagoya, Japan). Naringenin, (+)-catechin hydrate, soluble starch, dextrin, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, isomaltose, carboxymethyl cellose, α,α′-trehalose, α-cyclodextrin, γ-cyclodextrin, and dextran were purchased from Nacalai Tesque (Kyoto, Japan). Cellobiose was purchased from Sigma (St. Louis, MO). (−)-Epigallocatechin gallate (EGCG, Teavigo) was a product of DSM Nutritional Products (Basel, Switzerland). Esculetin was purchased from Tokyo Kasei Industries (Tokyo, Japan). Kaempferol and quercetin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Genistein and daidzein were purchased from Fujicco (Kobe, Japan). All other chemicals were of analytical grade.

Strains and Plasmids. *T. viride* JCM22452 was obtained from the IAM Culture Collection (Tokyo, Japan). *Saccharomyces cerevisiae* EH13-15 (*MAT*α, *trp1*) (*23*) was used as an expression host. The plasmid pCR2.1-TOPO (Invitrogen, Carlsbad, CA) was used as a cloning vector, and pYE22m (*24*) was used as an expression vector.

Cultivation and Preparation of Crude Enzyme. *T. viride* JCM22452 was grown in a medium containing 1% yeast extract, 1% peptone, and 2% dextrin. The cells were grown in a flask containing 900 mL of the medium with agitation at 30 °C for 3 days. The culture broth was filtered, and ammonium sulfate was added slowly to the resultant supernatant to a final concentration of 80% saturation. After the mixture was left for 30 min, the precipitate was collected by centrifugation and dissolved in 10 mL of 0.1 M sodium acetate buffer, pH 5.0. The resultant solution was designated the TR crude enzyme.

Phylogenetic Analysis of α-Amylase Homologues from Ascomycetes. Twenty-nine α-amylase homologues containing the α-amylase catalytic domain (GenBank accession no. PF00128) were extracted from the genomic databases of ascomycetes (*Aspergillus nidulans*, *Neurospora crassa*, *Magnaporthe grisea*, and *Fusarium graminearum*), and the phylogenetic tree was constructed using the CLUSTAL W multiple-alignment program using the neighbor-joining method.

Molecular Cloning of the Gene Encoding *T. viride* JCM22452 α-Amylase (TRa2). On the basis of the highly conserved region of ascomycetes α-amylase homologues contained in group II, degenerate PCR primers (AMY-f1: 5′-TAYTGYGGNGGNACNTTYAARGGNYT-3′, AMY-r1: 5′-TTYTCNACRTGYTTNACNGTRTCDAT-3′, AMY-r2: 5′-CCANARRTCYTCNCKRTTNGCNGGRTC-3′) were designed (Supporting Information Figure 1). Genomic DNA was prepared and purified from *T. viride* JCM22452 using a DNeasy plant Maxi Kit (Qiagen, Hilden, Germany). PCR was performed using the Ex Taq DNA polymerase (TAKARA BIO, Otsu, Japan) with the degenerate PCR primers and the genomic DNA as a template. The thermal cycling conditions were 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and then 72 °C for 10 min. The amplified fragments (∼1 kbp in length), termed partial *TRa2*, were cloned into TOPO-pCR2.1 (Invitrogen) and sequenced using ABI3100Avant (Applied Biosystems). Inverse PCR was performed using LA Taq DNA polymerase (TAKARA BIO) with the *T. viride* JCM22452 genomic DNA digested with *Hin*dIII or *Pst*I and self-ligated as a template and the primers TRa2-1 (5′-CCAACCTGGTATCTACATAC-3′) and TRa2-2 (5′-AGATGGCATCAAATCCCAT-3′) designed on the basis of the partial TRa2 DNA. The thermal cycling conditions were 94 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 66 °C for 15 min, and then 72 °C for 10 min. The amplified fragments, which were ∼5 kbp in length, were cloned and sequenced as described above. Total RNA was prepared from *T. viride* JCM22452 cells using the RNeasy Plant Mini Kit (Qiagen) and converted to cDNA using a SuperScript First-Strand Synthesis System for reverse transcriptase (RT) (Invitrogen, Carlsbad, CA) with a random hexamer. RT-PCR was performed using the cDNA as a template and the primers TRa2*Eco*RI-f (5′-GGAATTCATGAAGCTTCGATCCGCCGTCCC-3′; the underlined region indicates the *Eco*RI site) and TRa2XhoI-r (5′-CCGCTCGAGTTATGAAGACAGCAGCACAAT-3′; the underlined region indicates the *Xho*I site). The thermal cycling conditions were 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and then 72 °C for 10 min. The amplified fragment was cloned and sequenced as described above, and the resultant plasmid was designated pCRTRa2. To express the C-terminal in-frame fusion of TRa2 with a His_6 tag, PCR was performed using the pCRTRa2 as a template and the primer TRa2*Eco*RI-f and TRa2HisXhoI-r (5′-GCTCGAGTTAGTGGTGGTGGTGGTGTGAAGACAGCAGCAA-3′; the underlined region indicates the *Xho*I site). The thermal cycling conditions were 94 °C for 2 min, followed by 25 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, and then 72 °C for 10 min. The amplified fragment was cloned and sequenced as described above, and the resultant plasmid was designated pCRTRa2-His.

Expression and Purification of the Recombinant Enzyme. pCRTRa2 and pCRTRa2-His were digested with *Eco*RI and *Xho*I, and the resulting DNA fragments were ligated with a pYE22m vector that had been digested previously with *Eco*RI and *Sal*I to obtain the plasmid pYETRa2 and pYETRa2-His, respectively. pYETRa2 and pYETRa2-His were transformed into *S. cerevisiae* EH13-15, and the transformants were designated TRa2/EH13-15 and TRa2-His/EH13-15, respectively. TRa2/EH13-15 cells were precultured at 30 °C for 16 h in YPD medium. Ten milliliters of the preculture was then inoculated into 500 mL of the same medium at 30 °C for 3 days. The recombinant *S. cerevisiae* cells were removed by centrifugation. The supernatant was concentrated and desalted by ultrafiltration followed by buffer replacement in 100 mM sodium acetate buffer, pH 5.0, at 4 °C, and the resultant solution was designated TRa2 crude enzyme. TRa2-His/EH13−-15 cells were precultured at 30 °C for 16 h in an SD Trp dropout medium. Twenty milliliters of the preculture was then inoculated into 1 L of the same medium including 100 mM potassium phosphate buffer, pH 6.0, at 30 °C for 3 days. All subsequent operations were conducted at 0−4 °C. The recombinant *S. cerevisiae* cells were removed by centrifugation, and the supernatant was applied to a Ni^{2+}-Chelating Sepharose Fast Flow column (5 mL, GE Healthcare Bio-Sciences, Piscataway, NJ) equilibrated with buffer A (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, and 15 mM β-mercaptoethanol) containing 10 mM imidazole. The column was washed with the same buffer followed by elution of the enzyme with buffer A containing 200 mM imidazole. The eluted fraction showing transglycosylation activity was concentrated and desalted using VIVASPIN 30,000 MWCO (VIVASCIENCE, Hanover, Germany) followed by buffer replacement in buffer B (20 mM potassium phosphate, pH 7.4, 0.1% (w/v) 2-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and 15 mM β-mercaptoethanol). The enzyme solution was applied to a Resource Q column (1 mL, GE Healthcare Bio-Sciences) equilibrated with buffer B at a rate of 1.5 mL/min using the ÄKTA system (GE Healthcare Bio-Sciences). After the column had been washed with buffer B, the enzyme was eluted with a linear gradient of 0−600 mM NaCl in buffer B. The active fractions were combined, concentrated, and rechromatographed under the same conditions. The active fractions were combined, concentrated, and equilibrated with buffer C (20 mM potassium phosphate, pH 7.4, and 15 mM β-mercaptoethanol) as described above. The resultant solution was designated TRa2-His. The protein concentration was determined according to the Bradford method (*25*) using bovine serum albumin as a standard. SDS-PAGE was carried out in accordance with the method of Laemmli (*26*), and the proteins in the gels were visualized by silver staining. The TRa2 crude enzyme was used for glycosyl acceptor substrate specificity analysis, and the TRa2-His was used for stability and catalytic property analysis.

Enzyme Reactions. *Transglucosylation Activity of a Culture of T. viride JCM22452.* A culture of *T. viride* JCM22452 was filtered and concentrated 70-fold by ammonium sulfate precipitation, and the resultant precipitation was resuspended in 100 mM sodium acetate buffer, pH 5.0. One hundred microliters of the resultant enzyme solution was added to 3 mg of (+)-catechin and 10 mg of dextrin. After incubation at 50 °C for 24 h, the reaction was stopped by the addition of 900 μL of 0.1% trifluoroacetic acid. The enzyme products were analyzed by reversed-phase high-performance liquid chromatography (HPLC) on a column Develosil C30-UG-5 (4.6 × 150 mm, Nomura Chemical, Seto, Japan). The substrates and products were eluted with a linear gradient of 4.5−45% (v/v) CH_3CN containing 0.1% (v/v) trifluoroacetic acid for 20 min at a flow rate of 1 mL/min and were detected at 280 nm using an L-7455 diode array detector (Hitachi, Tokyo, Japan).

Glucosyl Donor Specificity for the Transglucosylation of Cellulase Onozuka RS and the TRa2 Crude Enzyme with (+)-Catechin as a Glucosyl Acceptor. The mixture of 3 mg of (+)-catechin hydrate, 10 mg of glucosyl donor substrate (soluble starch, dextrin, cyclodextrin, maltooligosaccharides, cellobiose, and the others), and 1.3 mg of TRa2 or 10 mg of Cellulase Onozuka RS was dissolved in 100 μL of 100 mM sodium acetate buffer, pH 5.0, and the reaction and analysis were carried out as described above.

Glucosyl Acceptor Specificity for the Transglucosylation of TRa2 Crude Enzyme. The mixture of 0.5 mM glucosyl acceptor (flavonoids and coumarin), 10 mg of dextrin, and 0.65 mg of TRa2 was dissolved in 100 μL of 100 mM sodium acetate buffer, pH 5.0. After incubation at 45 °C for 24 h, the reaction was stopped by the addition of 100 μL of 0.5% trifluoroacetic acid. The enzyme products were analyzed as described above. The respective relative activity of the glucosyl acceptors for EGCG were given as follows:

$$\text{relative activity (\%)} = \frac{\text{total area of glucosylated products/area of unreacted aglycone}}{\text{total area of glucosylated products/area of unreacted EGCG}} \times 100$$

The average value of the area was given by at least three experiments with deviations of 10% from the mean.

Enzyme Kinetics (Maltooligosaccharide Hydrolysis with TRa2-His). The standard reaction mixture (100 μL) consisted of 5 mM maltooligosaccharide, 20 mM sodium acetate buffer, pH 5.0, and the TRa2-His. The mixture without enzyme was preincubated at 35 °C for 10 min, and the reaction was started by the addition of enzyme. After incubation at 35 °C for 15 min, the reaction was stopped by heating at 100 °C for 5 min. The enzyme products were analyzed by isocratic normal-phase HPLC on a Supelcosil LC-NH_2 column (4.6 × 250 mm, Sigma). The substrates and products were eluted with 68% (v/v) CH_3CN at a flow rate of 1 mL/min and detected at a reflective index using an L-7490 RI detector (Hitachi). The number of the α-1,4-glycosidic linkage cleaved by the enzyme was designated N_{M3}, N_{M4}, N_{M5}, N_{M6}, and N_{M7} for maltotriose (M3), maltotetraose (M4), maltopentaose (M5), maltohexaose (M6), and maltoheptaose (M7), respectively. For example, in an initial velocity assay, because M3 was hydrolyzed to "one glucose" and "one M2" per cleavage, N_{M3} was equal to the number of M2 produced by hydrolysis. On the other hand, because an M4 was hydrolyzed to "one glucose" and "one M3" or "two M2" per cleavage, N_{M4} was equal to the sum of the number of M3 and half of the M2 produced by hydrolysis. With similar consideration for other substrates, N_{M3}, N_{M4}, N_{M5}, N_{M6}, and N_{M7} were calculated using the equations

$$N_{M3} = [M2]$$

$$N_{M4} = [M2]/2 + [M3]$$

$$N_{M5} = [M3] + [M4]$$

$$N_{M6} = [M3]/2 + [M4] + [M5]$$

$$N_{M7} = [M4] + [M5] + [M6]$$

where the brackets denote the number of the molecule.

The initial velocity assay of the hydrolysis activity for TRa2-His was carried out under steady-state conditions with various concentrations of substrates. Apparent K_m and V_{max} values for maltooligosaccharides were determined by fitting the initial velocity data of the number of the cleaved α-1,4-glycosidic linkage to a Michaelis−Menten equation by nonlinear regression analysis (*27, 28*).

Preparation of Glucosylated Products. The reactions for the preparation of glucosylated products were demonstrated as described below. Each fraction containing major product peaks in HPLC was collected and lyophilized, and the chemical structure of the resultant compounds used was identified by positive- or negative-mode time-of-flight mass (TOF-MS) spectrometry and NMR spectroscopy, as described previously (*29*).

(+)-Catechin Glucosides with Cellulase T Amano 4. (+)-Catechin (60 mg), soluble starch (200 mg), and Cellulase T Amano 4 (200 mg) were added to 2 mL of 100 mM sodium acetate buffer, pH 5.0, and the mixture was incubated with agitation at 50 °C for 3 days. After incubation, the insoluble substance was removed by centrifugation, and the products were purified by reversed-phase HPLC on a Develosil C30-UG-5 column (20 × 250 mm). The column was eluted with 18% (v/v) CH_3CN containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 4 mL/min with monitoring at 280 nm. The chromatogram is shown in **Figure 2a**.

EGCG Glucosides with Cellulase Onozuka RS. EGCG (120 mg), dextrin (400 mg), and Cellulase Onozuka RS (400 mg) were added to 3 mL of 100 mM sodium acetate buffer, pH 5.0, and the reaction and purification were carried out as described above. The column was eluted with 40% (v/v) methanol at a flow rate of 3 mL/min with monitoring at 280 nm. The chromatograph was shown in **Figure 2b**.

EGCG Glucosides with Pancelase BR. EGCG (3 g), dextrin (10 g), and Pancelase BR (5 g) were added to 100 mL of 100 mM sodium acetate buffer, pH 5.0, and the mixture was incubated with agitation at 50 °C for 4 h. After incubation, the insoluble material was removed by centrifugation, and the supernatant was adsorbed to a column of Sepharose LH20 (2.5 × 40 cm, GE Healthcare Bio-Sciences). The column was washed with 200 mL of H_2O and then eluted with 200 mL of 30% ethanol and 40% ethanol. The fraction containing the products was further purified as described above. The column was eluted with 27% (v/v) methanol containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 3 mL/min with monitoring at 280 nm. The chromatogram is shown in **Figure 2c**.

(+)-Catechin Glucosides with TRa2. (+)-Catechin (1.5 g) and dextrin (5 g) were added to 650 mg of TRa2 crude enzyme in 50 mL of 100 mM sodium acetate buffer, pH 5.0, and the mixture was incubated with agitation at 45 °C for 18 h. After incubation, the insoluble material was removed by centrifugation, and the supernatant was adsorbed to a column of Sepharose LH20 (2.5 × 20 cm). The column was washed with 120 mL of H_2O and then eluted with 240 mL of 10% ethanol. The fraction containing the products was further purified as described above. The column was eluted with 27% (v/v) methanol containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 3 mL/min with monitoring at 280 nm. The chromatogram is shown in **Figure 2e**.

EGCG Glucosides with TRa2. EGCG (600 mg) and dextrin (2 g) were added to 260 mg of TRa2 crude enzyme in 20 mL of 100 mM sodium acetate buffer, pH 5.0, and the mixture was incubated with agitation at 55 °C for 24 h. After incubation, the insoluble material was removed by centrifugation, and the supernatant was adsorbed to a column of Sepharose LH20 (1.5 × 30 cm). The column was washed with 100 mL of H_2O and then eluted with 100 mL of 10, 20, and 30% ethanol. The fraction containing the products was further purified as described above. The column was eluted with 36% (v/v) methanol containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 3 mL/min with monitoring at 280 nm. The chromatogram is shown in **Figure 2f**.

Measurement of the Stability, Solubility, and Taste of Glucosylated Products. To compare the thermal stability of EGCG glucoside with EGCG, the flavonoids (100 μM) were treated at pH 7.0 at 80 °C for 6 h. After the mixtures had been chilled on ice, the remaining flavonoids were determined by HPLC as described above. The remaining ratio (percent) was given as the ratio of the area of the flavonoid at 6 h to that at the starting point (0 h) as shown in **Table 2**.

To compare the water solubility of EGCG glucoside with EGCG, the flavonoids were mixed in 1 mL of distilled water at room temperature. After centrifugation of the mixture, the flavonoid concentrations (mg/mL) of the supernatant were determined by HPLC as described (**Table 2**).

These data were also given by at least three experiments with deviations of 10% from the mean.

The taste of EGCG glucoside was compared with that of EGCG. The flavonoids (440 μM) were dissolved in distilled water, and their taste values were measured using a Taste Sensing System SA402B (Intelligent Sensor Technology, Kanagawa, Japan). The analysis of taste of these flavonoids was entrusted to Taste & Aroma Strategic Research Institute Co. Ltd. (Kanagawa, Japan).

RESULTS

Identification of Glucosylated Products Obtained Using Commercial Cellulase Preparations from *T. viride*. As shown

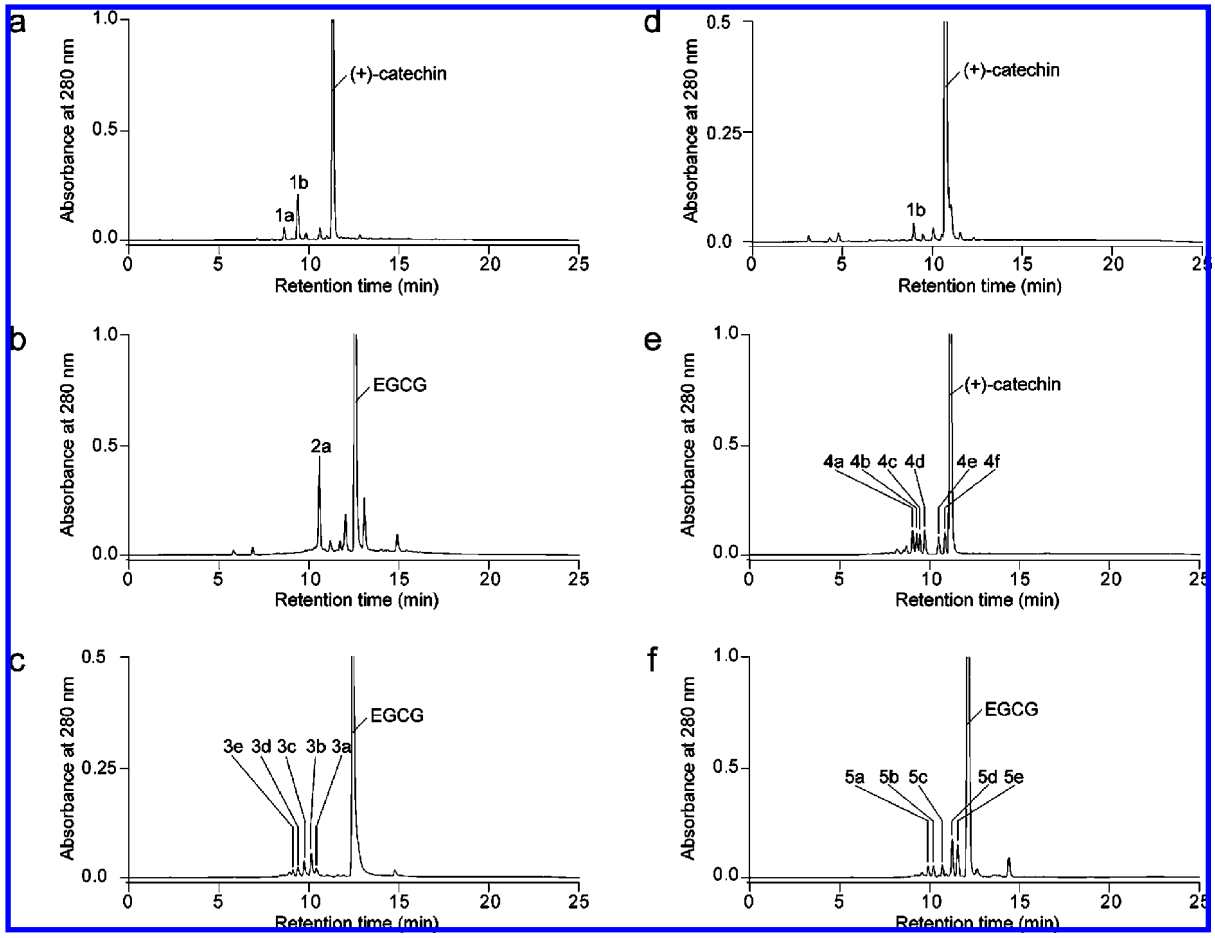

Figure 2. Transglucosylation activity of the enzymes from *T. viride*: (**a**, **d**) HPLC profile of reaction mixtures of Cellulase T Amano 4 (**a**) and culture of *T. viride* JCM22452 (**d**) with (+)-catechin and dextrin; (**b**, **c**) HPLC profile of reaction mixtures of Cellulase Onozuka RS (**b**) and Pancelase BR (**c**) with EGCG and dextrin; (**e**, **f**) HPLC profile of reaction mixtures of TRa2 with (+)-catechin (**e**) or EGCG (**f**) and dextrin.

Table 1. Kinetic Parameters of the Hydrolysis Activity for TRa2

substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
maltotriose	16 ± 6	8.2 ± 5.7	1.98
maltotetraose	127 ± 10	2.8 ± 0.5	45.2
maltopentaose	134 ± 4	2.3 ± 0.2	58.3
maltohexaose	230 ± 11	2.4 ± 0.3	97.4
maltoheptaose	274 ± 10	3.0 ± 0.3	92.6

in **Figure 2a**−**c**, some glucosylated products were observed by enzyme reaction with commercial cellulase preparations, such as Cellulase T Amano 4, Cellulase Onozuka RS, and Pancelase BR. These products were isolated and identified. The TOF-MS spectrometry and selected ^{1}H NMR spectroscopy data of each product are described in Supporting Information Table 1. On the basis of the coupling constant (J = 3−4 Hz, data not shown) of the anomeric hydrogen of these products, it was concluded that these products were α-glucosides. These data indicated that **1a**, **1b**, **2a**, **3a**, and **3b** were (+)-catechin 7-*O*-α-D-glucopyranoside, (+)-catechin 5-*O*-α-D-glucopyranoside, EGCG 5-*O*-α-D-glucopyranoside, EGCG 7-*O*-α-D-glucopyranoside, and EGCG 7-*O*-α-D-maltoside, respectively (**Figure 1**). Compounds **3c**, **3d**, and **3e** were speculated to be EGCG 7-*O*-α-D-maltotrioside, EGCG 7-*O*-α-D-maltotetraoside, and EGCG 7-*O*-α-D-maltopentaoside, respectively, on the basis of the TOF-MS and NMR data of **3a** and **3b**. To our knowledge, (+)-catechin 5-*O*-α-D-glucopyranoside, EGCG 5-*O*-α-D-glucopyranoside, and EGCG 7-*O*-α-D-maltoside are reported here for the first time, whereas (+)-catechin 7-*O*-α-D-glucopyranoside (*16, 17*) and EGCG 7-*O*-α-D-glucopyranoside (*12*) have been reported previously.

Glucosyl Donor Specificity. The glucosyl donor specificity of Cellulase Onozuka RS was examined with several oligosaccharides and polysaccharides using (+)-catechin as a glucosyl acceptor. The enzyme exhibited the transglucosylation activity in the case of using soluble starch, dextrin, γ-cyclodextrin, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose as a donor, but not in the case of using maltose, cellobiose, isomaltose, carboxymethyl cellulose, α,α′-trehalose, α-cyclodextrin, and dextran (data not shown). Moreover, a culture of *T. viride* JCM22452 showed transglucosylation activity for (+)-catechin with dextrin (**Figure 2d**). On the other hand, these cellulase preparations did not produce any (+)-catechin β-glucoside when cellobiose or carboxymethyl cellulose was used as a substrate.

Phylogenetic Analysis of Ascomycete α-Amylase Homologues and Cloning of the TRa2 Gene. Twenty-nine open reading frames (ORFs) containing the α-amylase catalytic domain were extracted, and homology alignment and phylogenetic analysis were performed with the CLUSTAL W program. A phylogenetic tree of α-amylase homologues from ascomycetes (*A. nidulans*, *N. crassa*, *M. grisea*, and *F. graminearum*) shows several groups (groups I−IV, **Figure 3**). On the basis of the highly conserved region of group II of the α-amylase homologues (Supporting Information Figure 1), degenerate PCR primers (AMY-1f, AMY-2r, and AMY-3r) were designed. Primers AMY-1f and AMY-2r or AMY-1f and AMY-3r were

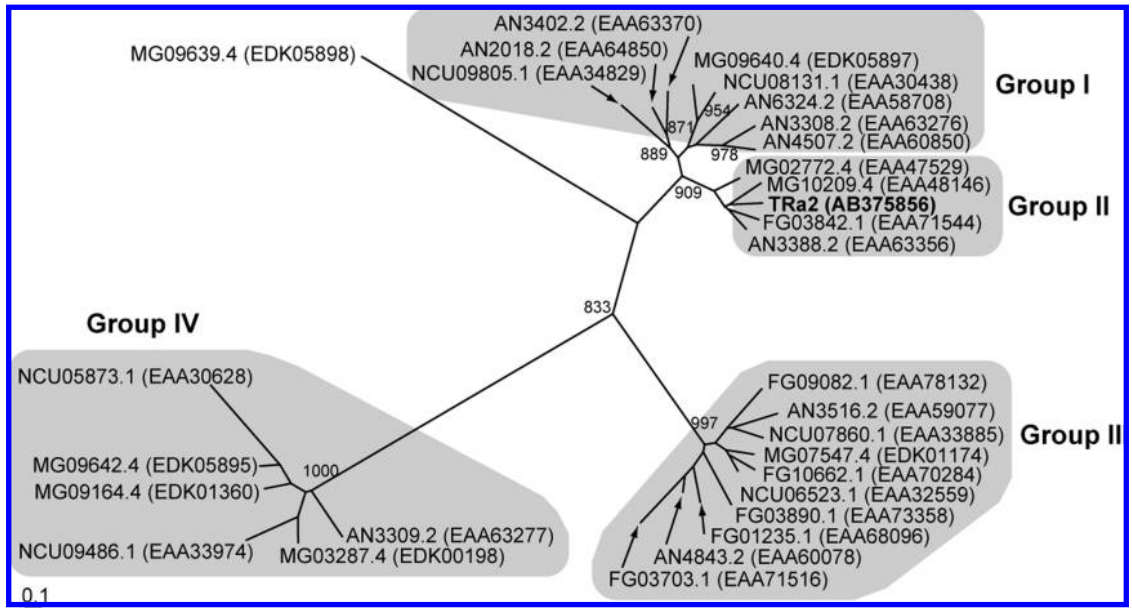

Figure 3. Phylogenetic analysis of *ascomycete* α-amylase. The sequences used for the alignment were primarily those of α-amylase homologues containing the α-amylase catalytic domain (GenBank accession no. PF00128) from genomic databases of ascomycetes (*A. nidulans*, *N. crassa*, *M. grisea*, and *F. graminearum*) and were classified into four groups (groups I−IV, gray shading). The phylogenetic tree was constructed using the CLUSTAL W multiple-alignment program using the neighbor-joining method and displayed using TreeView (*36*). Bar = 0.1 amino acid substitution/site. Numbers indicate bootstrap values >800. Parentheses indicate DDBJ/GenBankTM/EBI accession numbers of enzymes used for alignment. *Ascomycete* α-amylase homologues, except *A. nidulans*, *N. crassa*, *M. grisea*, and *F. graminearum*, are indicated in italics. The organisms from which each ORF is derived are as follows: AN2018.2, AN3308.2, AN3309.2, AN3388.2, AN3402.2, AN3516.2, AN4507.2, AN4843.2, and AN6324.2, *A. nidulans*; FG01235.1, FG03703.1, FG03842.1, FG03890.1, FG09082.1, and FG10662.1, *F. graminearum*; MG02772.4, MG03287.4, MG07547.4, MG09164.4, MG09639.4, MG09640.4, MG09642.4, and MG10209.4, *M. grisea*; NCU05873.1, NCU06523.1, NCU07860.1, NCU08131.1, NCU09486.1, and NCU09805.1, *N. crassa*; TRa2, *T. viride* JCM22452.

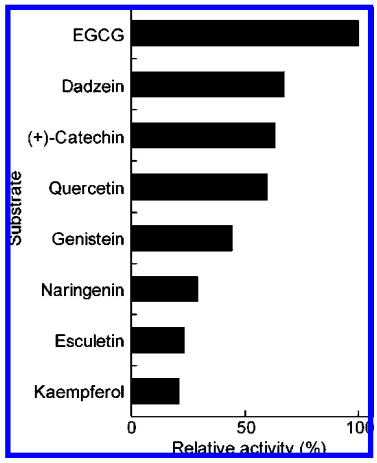

Figure 4. Substrate specificity of the glucosyl-transfer reaction of TRa2. Relative activities for flavonoids and coumarin are shown (activity for EGCG = 100%).

used in PCR with the *T. viride* JCM22452 genomic DNA as a template, yielding PCR products of 0.6 or 1.0 kbp, respectively. When two PCR products were compared, the former was found to be identical to a part of the latter. To determine the genomic sequence including the full-length *TRa2* DNA, inverse PCR was carried out using primers TRa2-1 and TRa2-2 designed on the basis of the partial *TRa2* DNA sequence. Furthermore, TRa2*Eco*RI-f and TRa2*Xho*I-r, designed on the basis of the full-length *TRa2* DNA, were used in RT-PCR to obtain the full-length *TRa2* cDNA sequence.

The full-length *TRa2* cDNA contained an ORF of 1389 bp encoding 463 amino acid residues (DDBJ/GenBank/EBI accession no. AB375856). In comparison with the *TRa2* cDNA sequence, the *TRa2* gene in the genomic DNA contained two introns. Within the deduced amino acid sequence of TRa2, there is a deduced 20-residue signal sequence at the N terminus, which would be probably removed in conjunction with the translocation of TRa2 through the cytoplasmic membrane (*30*).

Table 2. Solubility and Stability of EGCG and EGCG Glucoside 5G[a]

sample	solubility in water (mg/mL)	remaining amount after heat treatment (%)
EGCG	39.6	50
EGCG 5G	>208	77

[a] EGCG 5G, EGCG 5-*O*-α-D-glucopyranoside.

The deduced amino acid sequence of TRa2 contains four highly conserved regions related to the catalytic and substrate-binding sites in the α-amylase family and three amino acid residues (Asp, Glu, and Asp) as catalytic sites (Supporting Information Figure 1) (*31*). The sequence showed identities with α-amylase homologues classified in group II (**Figure 2**): AN3388.2 (61% identity) from *A. nidulans*, MG10209.4 (61% identity) and MG02772.4 (55% identity) from *M. grisea*, and FG03842.1 (58% identity) from *F. graminearum*.

Expression and Purification of TRa2. TRa2 was expressed under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter and terminator in *S. cerevisiae* EH13-15 cells, and its α-amylase activity was found in the extra- and intracellular fractions. In contrast, *S. cerevisiae* EH13-15 cells harboring pYE22m, which were used as a negative control, showed no α-amylase activity. On the other hand, TRa2-His, the C-terminal in-frame fusion of TRa2 with the His_6 tag, was expressed for convenient purification. TRa2-His could be purified to homogeneity from the culture supernatant of *S. cerevisiae* EH13-15 cells harboring pYETRa2-His after three purification steps (see Experimental Procedures).

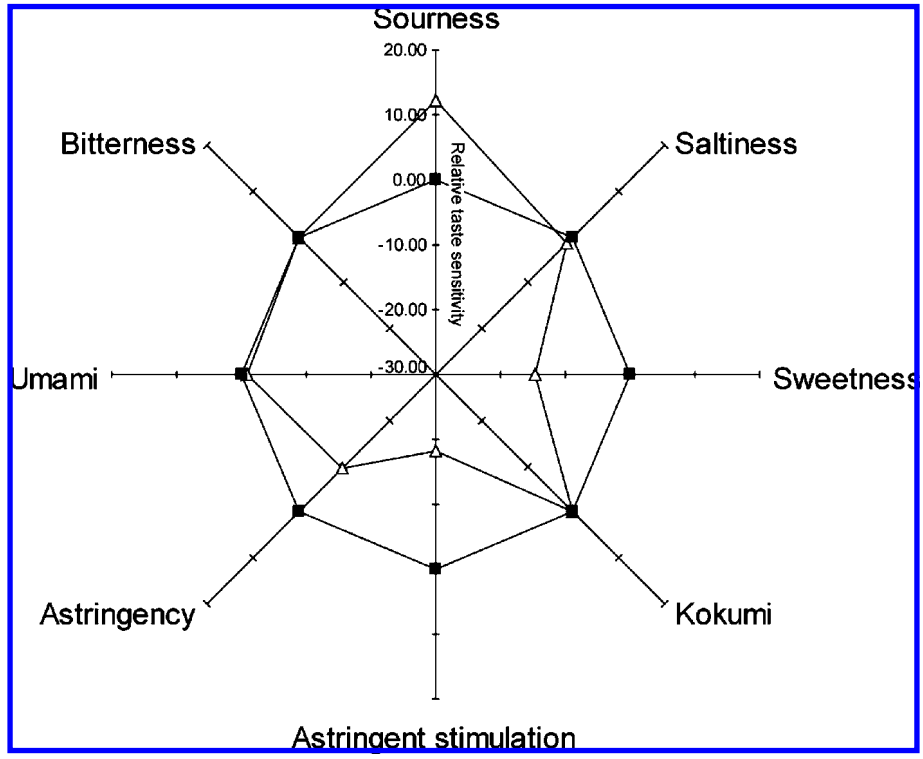

Figure 5. Taste sensitivities of EGCG (solid circle) and EGCG 5-*O*-α-D-glucopyranoside (open circle). Relative taste sensitivities for these flavonoids are shown (the value for EGCG = 0) as the average values of four independent determinations. The 20% difference in concentration between standard and sample solutions is converted to a graduation of relative taste sensitivity; that is, relative taste sensitivity equals $\log_{1.2}$ (the difference in concentration between standard and sample solutions). "Kokumi" is a taste-enhancing quality and has been described variously as continuity, mouthfulness, mouthfeel, and thickness.

Characterization of TRa2. *Glucosyl Acceptor Substrate Specificity of the Glucosyl-Transfer Reaction.* The substrate specificity of TRa2 was examined with a wide variety of flavonoids and coumarin using dextrin as the glucosyl donor. The enzyme showed the highest activity with EGCG (**Figure 4**). It also showed appreciable activities toward the following flavonoids and coumarin: daidzein (relative activity for EGCG, 67%), (+)-catechin (63%), quercetin (60%), genistein (44%), naringenin (29%), esculetin (24%), and kaempferol (21%). HPLC analysis showed that the reaction of TRa2 with esculetin yielded a single transfer product, whereas the reaction with other substrates yielded several.

Enzymatic Properties. The purified TRa2-His could hydrolyze *p*-nitrophenyl α-D-maltoside but not *p*-nitrophenyl α-D-glucoside (data not shown). Similarly, it could hydrolyze M3, M4, M5, M6, and M7 (Supporting Information Figure 2a−e) but not M2. Moreover, the reaction of purified TRa2-His with M3 yielded M5, not M4, as a glucosyl-transfer product, together with both glucose and M2 as hydrolyzed products (Supporting Information Figure 2a). It was concluded that M5 was produced by transferring a non-reducing-terminal maltose unit of M3 to another M3 and that the enzyme also cleaved the non-reducing-terminal maltosyl bond of M3 to form glucose and M2. Hydrolysis of maltooligosaccharides with TRa2-His gave M2 and the rest of each substrate (Supporting Information Figure 2a−e) as the major products. The k_{cat} and K_m values for M3 at pH 6.0 and 35 °C were 8−17 times lower and 2.7−3.6 times higher, respectively, than those for M4−M7 (**Table 1**). Thus, the calculated specificity constant (k_{cat}/K_m) for M3 was 20−50 times lower than that for M4−M7. Moreover, the k_{cat} values for M6 and M7 were about 2 times higher than that for M4 and M5, whereas the K_m values for M4−M7 were equivalent.

The optimum pH and temperature of the purified TRa2-His for the hydrolysis of maltotetraose were at around pH 5.0 and 55 °C, respectively, and the enzyme was stable at pH 4.0−5.5 (at 4 °C for 16 h) and below 40 °C (at pH 5.0 for 1 h).

Structural Study of the Reaction Products of TRa2-Catalyzed Glucosyl Transfer to (+)-Catechin and EGCG. As shown in **Figure 2e**,**f**, some glucosylated products were observed by enzyme reaction with TRa2. These products were also isolated and identified. TOF-MS spectrometry and selected ^{1}H NMR spectroscopy data of each product are described in Supporting Information Tables 2 and 3. On the basis of the coupling constant (J = 3−4 Hz, data not shown) of the anomeric hydrogen of these products, it was concluded that these products were α-glucosides. These data showed that **4a**, **4b**, **4c**, **4d**, **4e**, and **4f** were (+)-catechin 5-*O*-α-D-maltoside, (+)-catechin 5-*O*-α-D-glucopyranoside (the same as **1b**), (+)-catechin 4′-*O*-α-D-maltoside, (+)-catechin 4′-*O*-α-D-glucopyranoside, (+)-catechin 3′-*O*-α-D-maltoside, and (+)-catechin 3′-*O*-α-D-glucopyranoside, respectively (**Figure 1**). Similarly, **5a**, **5c**, **5d**, and **5e** were EGCG 7-*O*-α-D-maltoside (the same as **3b**), EGCG 3′-*O*-α-D-maltotrioside, EGCG 3′-*O*-α-D-maltoside, and EGCG 3′-*O*-α-D-glucopyranoside, respectively (**Figure 1**). The results of HPLC and MS generated speculation that product **5b** could be a mixture of EGCG 7-*O*-α-D-glucopyranoside and EGCG 3′-*O*-α-D-maltotetraoside. To our knowledge, (+)-catechin 5-*O*-α-D-maltoside and (+)-catechin 4′-*O*-α-D-maltoside are reported here for the first time, whereas (+)-catechin 3′-*O*-α-D-glucopyranoside (*1*,*8*,*13*), (+)-catechin 4′-*O*-α-D-glucopyranoside (*17, 18*), EGCG 3′-*O*-α-D-glucopyranoside, (+)-catechin 3′-*O*-α-D-maltooligoglycoside, and EGCG 3′-*O*-α-D-maltooligoglycoside (*32*) have been reported previously.

Studies of the Stability, Solubility, and Taste of Glucosylated EGCG. The water solubility, thermal stability, and taste

of EGCG glucoside were examined and compared with those of EGCG. EGCG 5-*O*-α-D-glucopyranoside was >5 times more soluble in water than EGCG (**Table 2**), as reported previously (*8, 12*). Moreover, EGCG 5-*O*-α-D-glucopyranoside was thermally more stable than EGCG (**Table 2**). The taste of the aqueous solution of EGCG 5-*O*-α-D-glucopyranoside was compared with that of EGCG. The results of taste analysis using a multichannel taste sensor (*33, 34*) showed that the astringency and astringent stimulation of the EGCG 5-*O*-α-D-glucopyranoside solution were markedly lower than those of EGCG (**Figure 5**). Moreover, sensory analysis by panelists gave the same results as the taste analysis using the multichannel taste sensor (data not shown).

DISCUSSION

Commercial cellulase preparations, such as Cellulase T Amano 4, Cellulase Onozuka RS, and Pancelase BR, are typically used to hydrolyze β-linkage glucosides such as cellulose, naturally occurring flavonoid glucosides, and so on. Even if they possibly contain secretory α-amylases, which are produced in the cultured *T. viride* (*21, 22*), it has not been reported so far that they exhibit α-linkage glucose-transfer reaction.

In this study, it was found that the commercial enzyme preparations from *T. viride* showed transglucosylation activity toward (+)-catechin and EGCG using dextrin as a glucosyl donor (**Figure 2a**−**c**). The transglucosylation reaction of these enzymes occurred only using α-1,4-glucopoly- and oligosaccharides such as dextrin and maltooligosaccharides, not β-1,4-glucopoly(oligo)saccharides such as cellulose. It was suggested that the enzymes have α-amylase activity (endotype α-glucosidase), exhibiting high specificity for α-1,4-linkages. Moreover, it was found that the *T. viride* JCM22452 enzyme preparation showed transglucosylation activity toward (+)-catechin by culturing with dextrin (**Figure 2d**). Thus, *T. viride* (JCM22452) secretes at least one α-amylase that exhibits transglucosylation activity toward (+)-catechin and EGCG.

Then, to obtain the enzyme exhibiting transglucosylation activity toward (+)-catechin and EGCG, the cDNA encoding α-amylase was isolated from *T. viride* JCM22452, designated TRa2, by homology cloning. TRa2 expressed in *S. cerevisiae* EH13-15 exhibited transglucosylation activity toward flavonoids including (+)-catechin and EGCG. The active site of the α-amylase family enzymes is considered to have some subsites, each of which is capable of interaction with one glucose residue of the substrate (*35*). When maltooligosaccharides were used as the substrate, TRa2 showed M2-transfer activity, for example, producing M5 from M3 as a glucosyl-transfer product, and hydrolyzed each substrate to M2 and the rest, suggesting that TRa2 needs at least two glucose residues in the nonreducing terminal to hydrolyze glucooligo- or glucopolysaccharide; that is, it is essential for subsite −2 of the enzyme to be occupied by a glucose residue, demonstrating that TRa2 was an endotype amylase, not an exotype amylase. Moreover, the k_{cat} and K_m for M3 were lower and higher, respectively, than those of M4−M7, and the k_{cat} for M6 and M7 were higher than those for M4 and M5, although the K_m values for M4−M7 were equivalent (**Table 1**). These results also indicated that interaction with a glucose residue in subsite +2 of TRa2 could increase the affinity for the substrate and enhance the hydrolytic activity and that further interaction with other subsites could not increase the affinity for the substrate, although the hydrolytic activity may have been increased.

The commercial enzyme preparations from *T. viride* and TRa2 produced five novel glucosides, (+)-catechin 5-*O*-α-D-glucopyranoside, (+)-catechin 5-*O*-α-D-maltoside, (+)-catechin 4′-*O*-α-D-maltoside, EGCG 5-*O*-α-D-glucopyranoside, and EGCG 7-*O*-α-D-maltoside, in addition to some known glucosides. These (+)-catechin and EGCG oligoglucosides are expected to show higher intestinal absorbance than its monoglucoside, as well as quercetin oligoglycosides higher than quercetin monoglucoside (isoquercitrin) (*14*). The reaction of TRa2 with (+)-catechin preferentially yielded 3′- and 4′-*O*-glucosides, whereas that of the commercial cellulase preparations mainly yielded 5- and 7-*O*-glucosides. These results suggest that the main α-glucosidase contained in the commercial cellulase preparations was different from TRa2 because of the difference in the glucosylated positions for (+)-catechin. Furthermore, it is considered that TRa2 could not transfer the monoglucosyl residue to an acceptor from the results of the hydrolysis of maltooligosaccharides (Supporting Information Figure 2). Therefore, it was presumed that these obtained monoglucosides were produced by hydrolyzing the generated oligoglucosides. EGCG 5-*O*-α-D-glucopyranoside, one of the glucosides obtained by the commercial enzyme preparations from *T. viride* and TRa2, showed higher solubility and heat stability (**Table 2**) and lower astringency and astringent stimulation than its aglycone (**Figure 5**), suggesting that EGCG glucosides represent an improvement over EGCG as a food additive.

Considering that the genus *Trichoderma* is known to secrete several starch-acting hydrolases, such as glucoamylase and α-amylase (*21, 22*), TRa2 exhibiting a transglucosylation activity toward (+)-catechin and EGCG may be expressed only at low levels in the culture conditions of commercial enzyme preparations from *T. viride*. However, this is the first elucidation of a protein expressed from a cDNA obtained from *T. viride* JCM22452, TRa2, which showed transglucosylation activity toward (+)-catechin and EGCG. These findings make possible the large-scale preparation of this enzyme, which is able to glucosylate a wide range of natural flavonoids.

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Supporting Information Available: TOF-MS spectrometry and selected ^{1}H NMR spectroscopy data of (+)-catechin and EGCG glycosides, alignment of the deduced amino acid sequences of *ascomycete* α-amylase homologues, and time course data of hydrolysis of maltooligosaccharides with TRa2. This material is available free of charge via the Internet at http://pubs.acs.org.

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