Two Novel Glycoside Hydrolases Responsible for the Catabolism of Cyclobis-(1→6)-α-nigerosyl*

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The actinobacterium Kribbella flava NBRC 14399T produces cyclobis-(1→6)-α-nigerosyl (CNN), a cyclic glucotetraose with alternate α-(1→6)- and α-(1→3)-glucosidic linkages, from starch in the culture medium. We identified gene clusters associated with the production and intracellular catabolism of CNN in the K. flava genome. One cluster encodes 6-α-glucosyltransferase and 3-α-isomaltosyltransferase, which are known to coproduce CNN from starch. The other cluster contains four genes annotated as a transcriptional regulator, sugar transporter, glycoside hydrolase family (GH) 31 protein (Kfla1895), and GH15 protein (Kfla1896). Kfla1895 hydrolyzed the α-(1→3)-glucosidic linkages of CNN and produced isomaltose via a possible linear tetrasaccharide. The initial rate of hydrolysis of CNN (11.6 s⁻¹) was much higher than that of panose (0.242 s⁻¹), and hydrolysis of isomaltotriose and nigerose was extremely low. Because Kfla1895 has a strong preference for the α-(1→3)-isomaltosyl moiety and effectively hydrolyzes the α-(1→3)-glucosidic linkage, it should be termed 1,3-α-isomaltosidase. Kfla1896 effectively hydrolyzed isomaltose with liberation of β-glucose, but displayed low or no activity toward CNN and the general GH15 enzyme substrates such as maltose, soluble starch, or dextran. The Kcat/Km for isomaltose (4.81 ± 0.18 s⁻¹ mm⁻¹) was 6.9- and 19-fold higher than those for panose and isomaltotriose, respectively. These results indicate that Kfla1896 is a new GH15 enzyme with high substrate specificity for isomaltose, suggesting the enzyme should be designated an isomaltose glucohydrolase. This is the first report to identify a starch-utilization pathway that proceeds via CNN.

Cyclobis-(1→6)-α-nigerosyl (CNN), also known as cycolaternan or cyclic tetrasaccharide, is a cyclic glucotetraose with alternate α-(1→6)- and α-(1→3)-glucosidic linkages (Fig. 1). This cyclic sugar was first produced from alternan, an alternating linear α-(1→6)/α-(1→3)-glucan, by treatment with a glycanase termed alternanase, which is produced by Bacillus sp. NRRL B-21195 (1). Nishimoto and co-workers (2–4) subsequently identified three bacterial species (Sporosarcina globispora C11 and N75, formerly known as Bacillus globisporus, and Arthrobacter globiformis A19) that generate CNN from starch. Their ability to produce CNN is derived from two extracellular glycosyltransferases: 6-α-glucosyltransferase (6-GT) and 3-α-isomaltosyltransferase (IMT). 6-GT catalyzes α-(1→6)-glucosyl transfer to the nonreducing terminal glucose unit of α-(1→4)-glucan (GLn) and produces α-glucosyl-(1→6)-GLn (α-isomaltotetraosy-GLn). IMT utilizes α-isomaltotetraosyl-GLn as both donor and acceptor substrate and catalyzes the α-(1→3) transfer of the α-isomaltosyl moiety of one substrate to the other, resulting in α-isomaltosyl-(1→3)-α-isomaltotetraosyl-GLn formation. Furthermore, IMT catalyzes the intramolecular cyclization of α-isomaltotetraosyl-(1→3)-α-isomaltotetraosyl-GLn, and eventually generates CNN. Currently, it is known that Bacillus sp. NRRL B-21195 produces CNN from maltooligosaccharides using the alternanase and a 6-GT (5, 6). Gene clusters encoding 6-GT and IMT were identified in S. globispora C11 and N75, and A. globiformis A19 (3, 4, 7). Their 6-GT and IMT share high sequence similarity with related enzymes found among the bacterial species. All these enzymes are members of glycoside hydrolase family (GH) 31, which is a sequence-based enzyme classification (8). These studies imply that a common starch metabolic pathway that proceeds via CNN, in which 6-GT and IMT play crucial roles, had been distributed in some bacterial species beyond the genus.

Although CNN-producing enzymes have been identified in some bacteria, whether and how the bacterial species utilize CNN as a carbon source is unclear. Kim et al. (9) reported the purification and characterization of a CNN-degrading enzyme from a lysate of Bacillus sp. NRRL B-21195. This intracellular enzyme hydrolyzed CNN and produced isomaltose via the intermediate α-isomaltotetraosyl-(1→3)-isomaltose. Thus, the authors suggested that CNN is imported into the cells in its cyclic form, as well as cyclomaltoolactritins, which are the most widely known cyclic glucans (10). However, the complete CNN degradation pathway is yet unclear because no gene encoding a CNN-degrading enzyme or related proteins, such as a transporter or an isomaltose-degrading enzyme, has been identified.

In the present study, we revealed that the actinobacterium Kribbella flava NBRC 14399T extracellularly produces CNN from starch, and intracellularly degrades CNN into glucose via isomaltose. K. flava is a Gram-positive, aerobic, and mesophilic actinomycete isolated from soil or scabby potato, and its genome has been completely sequenced (11). This enabled us to...
identify two gene clusters related to CNN production and CNN degradation, respectively, in the genome. CNN degradation is catalyzed by two novel glycoside hydrolases that belong to GH31 and GH15, respectively. This is the first report to demonstrate this unique pathway, which metabolizes starch to glucose via CNN.

**Results**

Production and Degradation of CNN by *K. flavida*—*K. flavida* was cultured using soluble starch as the sole carbon source. The bacterium accumulated some oligosaccharides in the culture supernatant, one of which was a glucoamylase-resistant carbohydrate (Fig. 2A). Its molecular weight was estimated to be 648.2 using electrospray ionization (ESI)-MS analysis, and all chemical shifts observed in the $^1$H and $^{13}$C NMR spectra corresponded to those of CNN (1) (data not shown). These results demonstrate that this oligosaccharide was CNN.

The time courses of growth ($A_{600}$) and CNN concentration in the culture supernatant were monitored (Fig. 2B). The $A_{600}$ increased during the first 72 h of cultivation and then was decreased upon further cultivation. CNN was not observed in the culture medium until 72 h of cultivation, but further cultivation increased the accumulation of CNN. These observations indicate that *K. flavida* accumulated CNN in the death phase. The carbohydrate assimilation test showed that *K. flavida* grew well on CNN at the same level as on glucose and maltose (Fig. 2C), meaning that CNN is a favorable carbon source for this bacterium.

To ascertain whether *K. flavida* possesses CNN-degrading activity, a cell-free lysate, and a membrane fraction were reacted on CNN (Fig. 2D). Although the membrane fraction did not degrade CNN, the cell-free lysate produced isomaltose, glucose, and an oligosaccharide that may be a linearized CNN. These results demonstrate that *K. flavida* intracellularly catabolizes CNN via isomaltose.

**Candidate Gene Clusters Related to the Production and Degradation of CNN**—A search for genes in the *K. flavida* genome that encode proteins sharing similarity with IMT of *A. globiformis* A19 (UniProtKB accession: Q6BD67) was performed using the protein BLAST. Based on the search results, we focused on two genetic loci: *Kfla* _4052_ and *Kfla* _1895_.

*Kfla* _4052_ appears to form a gene cluster with *Kfla* _4051_ and *Kfla* _4053_ (Fig. 3A). The 1,108-amino acid GH31 protein encoded by *Kfla* _4052_, designated *Kfla*4052, shares 79.0% similarity (with 0.4% gaps; calculated using the EMBoss Water pairwise sequence alignment tool (12)) with the IMT of *A. globiformis* A19. The 948-amino acid protein encoded by *Kfla* _4051_, designated *Kfla*4051, shares high (78.3%) sequence similarity with the GH31 protein 6-GT of *A. globiformis* A19. The upstream gene (*Kfla* _4053_) was annotated as encoding a transcriptionsal regulator: repressor, open reading frame, kinase (ROK) family protein. Both *Kfla*4051 and *Kfla*4052 were predicted to possess a signal peptide, indicating that they are extracellular enzymes.

*Kfla* _1895_ appears to form another gene cluster with *Kfla* _1896_ to *Kfla* _1900_ (Fig. 3B). The 723-amino acid GH31 protein encoded by *Kfla* _1895_, designated *Kfla*1895, shares similarities with the IMT of *A. globiformis* A19 (46.2% similarity with 17.3% gaps) as well as other IMTs and *Kfla*4052. These enzymes were predicted to have an extra domain, a family-35 carbohydrate-binding module (CBM35) (8), at the C-terminal side of the catalytic domain, whereas *Kfla*1895 was predicted to be a single-domain protein. Its upstream gene, *Kfla* _1896_, was annotated as encoding a family-15 glycoside hydrolase. However, this 385-amino acid enzyme, *Kfla*1896, shared very weak similarity with GH15 enzymes, such as the glucoamylase of *Thermoactinomyces vulgaris* R-47 (37.1%) and the α,α-trehalase of *Mycobacterium smegmatis* str. MC2 155 (37.6%) because of high gap scores (25.2 and 15.4%, respectively). Both *Kfla*1895 and *Kfla*1896 were predicted to be intracellular pro-

![Cyclobis-(1→6)-α-nigerosyl](image-url)
Two Novel Glycosidases of GH31 and GH15 Degrade CNN

(A) 
\[ \text{Kfla}_{4051} \quad \text{Ag}_{-6GT} : 3.451 \quad \text{SpN75}_{-6GT} : 642 \quad \text{Cl}_{-4GT} : 555 \]
\[ \text{Kfla}_{4052} \quad \text{Ag}_{-IMT} : 3.968 \quad \text{SpCl11}_{-IMT} : 1.167 \quad \text{SpN75}_{-IMT} : 3.160 \]

(B) 
\[ \text{Kfla}_{1895} \quad \text{Ag}_{-IMT} : 1.207 \quad \text{SpCl11}_{-IMT} : 1.134 \quad \text{SpN75}_{-IMT} : 1.121 \quad \text{Kfla4052} : 1.104 \]

(C) 
\[ \text{Culture (h)} \]
\[ \text{Kfla}_{4051} \]
\[ \text{Kfla}_{4052} \]
\[ \text{Kfla}_{1895} \]
\[ \text{Kfla}_{1896} \]

FIGURE 3. Analysis of candidate genes. A and B, two focused gene clusters containing Kfla_{4052} and Kfla_{1895}, respectively. The values below each gene indicate the pairwise alignment score calculated using EMBOSS Water (12). The enzyme abbreviations and their UniProtKB accessions as follows: Ag_{-6GT}, 6-GT of A. globiformis A19 (Q6BD05); SpN75_{-6GT}, 6-GT of S. globispora N75 (Q84IO2); Cl_{-4GT}, 4-GT, oligosaccharide-α,1,4-transglucosylase of Cellvibrio japonicus Ueda107 (B3PE65); Ag_{-IMT}, IMT of A. globiformis A19 (Q6BD07); SpCl11_{-IMT}, IMT of S. globispora C11 (Q8R0V0); SpN75_{-IMT}, IMT of S. globispora N75 (Q84IO3); Tv_{-GA}, glucoamylase of T. vulgaris R-47 (Q9KR22); Ms_{-Tre}, α,α-trehalase of M. smegmatis str. MC2 155 (AOR099); Sh_{-GA}, glucoamylase of Streptomyces hygroscopicus subsp. limoneus KCCM 11405 (Q15JF7). C, gene expression analysis by semiquantitative RT-PCR. The 16S rRNA encoded by Kfla_{R0040} was used as a reference gene.

Teins because there was no possible signal peptide. Putative genes encoding a sugar ATP-binding cassette (ABC) transporter and an ROK family protein exist in their upstream regions.

The expression levels of Kfla_{4051}, 4052, 1895, and 1896 in K. flavida cells during CNN production were analyzed by semi-quantitative RT-PCR (Fig. 3C). All of their transcripts were present at detectable levels in cells cultured for 48 h or more. The expression levels of Kfla_{1895} and Kfla_{1896} were maintained during 168 h of culture, but those of Kfla_{4051} and Kfla_{4052} decreased at 168 h compared with their levels from 48 to 96 h.

Enzymatic Properties of Kfla4052, Kfla1895, and Kfla1896—Kfla4051, Kfla4052, Kfla1895, and Kfla1896 were expressed as recombinant proteins in Escherichia coli. All of the recombinant enzymes, except Kfla1895, were produced in a soluble form. Kfla4052, Kfla1895, and Kfla1896 were purified and characterized.

Kfla4052 was reacted on 20 mM panose (α-isomaltosyl-(1→4)-glucose) and the products were analyzed using TLC (Fig. 4A). The enzyme initially produced glucose and an oligosaccharide with a retention factor similar to that of maltotetraose. Subsequently, the reaction mixture accumulated CNN, indicating that Kfla4052 is a typical IMT. The oligosaccharide should be α-isomaltosyl-(1→3)-α-isomaltosyl-(1→4)-glucose. The activity of Kfla4052, defined using the rate of glucose release from 10 mM panose, was enhanced 18-fold in the presence of 1 mM CaCl₂. The activity was the highest at pH 6.6, and the enzyme was stable at pH 3.2 to 10.1 and at <55 °C.

Kfla1895 was reacted on 10 mM CNN and the products were analyzed using TLC (Fig. 4B, lanes 1–5). The enzyme initially produced a possible linearized CNN (its spot appears under that of CNN) and subsequently accumulated isomaltose. The initial rate for hydrolysis of 10 mM CNN, determined by an increase in the concentration of reducing ends, was 11.6 s⁻¹. Kfla1895 activity was the highest at pH 7.9, and the enzyme was stable between pH 6.8 and at least pH 10.7, and at <41 °C. Kfla1895 also hydrolyzed panose into glucose and isomaltose (data not shown); however, the initial rate of panose hydrolysis was 2% of that of CNN, and those of isomaltotriose and nigerose were vanishingly low (Table 1). The s-v plots for the hydrolysis of CNN were fitted to the Michaelis–Menten equation (Fig. 4C) and the kinetic parameters were determined (Table 1). The k_cat and K_m values for isomaltose were determined to be 22.3 ± 1.7 s⁻¹ and 7.63 ± 1.38 mM, respectively. The k_cat/K_m for CNN (2.97 ± 0.34 s⁻¹ mM⁻¹) was 57-fold higher than for panose (Table 2).

The sequence comparison with other GH31 enzymes revealed that two conserved regions, regions A and B, including the catalytic nucleophile and acid/base Asp residues, respectively (13), were also observed in Kfla1895 (Fig. 5A). The site-directed mutations of Asp⁻⁴⁵¹ and Asp⁻⁵¹⁶ of Kfla1895 to Ala resulted in complete loss of the CNN hydrolyzing activity (Fig. 5B). These results implied that Asp⁻⁴⁵¹ and Asp⁻⁵¹⁶ of Kfla1895 were, respectively, catalytic nucleophile and acid/base as found for other GH31 enzymes.

Kfla1896 was reacted on the reaction products from Kfla1895-catalyzed CNN hydrolysis, and the products of this reaction were analyzed using TLC (Fig. 4B, lanes 6 and 7). Kfla1896 completely hydrolyzed isomaltose to glucose and generated another oligosaccharide having a retention value similar to that of panose. Substrate specificity analysis revealed that Kfla1896 displays the highest reaction rate with isomaltose, lower rates with panose and isomaltotriose, but almost no activity with other α-glucobiases (trehalose, kojibiose, nigerose, and maltose), CNN, or polysaccharides (dextran and soluble starch) (Table 1). The isomaltose-hydrolyzing activity was the highest at pH 6.7, and the enzyme was stable between pH 6.5 and at least pH 11.2, and at <35 °C. All s-v plots for the hydrolysis of isomaltose, panose, and isomaltotriose were fitted to the Michaelis-Menten equation (Fig. 4D) and their kinetic parameters were determined (Table 2). Kfla1896 displayed a K_m value for isomaltose that was similar to those of its other substrates, but a 10-fold higher k_cat value for isomaltose than those.
Thus, Kfla1896 exhibited a $k_{cat}/K_m$ value for isomaltose that was 6.9- and 19-fold higher than those for panose and isomaltotriose, respectively. The anomeric configuration analysis of product showed that Kfla1896 produced the $\beta$-anomer of glucose (data not shown). Furthermore, Kfla1896 possessed two conserved regions among GH15 enzymes, regions III and V, including the catalytic acid and base glutamic acid residues, respectively (14), and the substitutions of Glu178 and Glu335 of Kfla1896 by Ala markedly decreased or lost the activity (Fig. 5, C and D). These results indicated that the catalytic mechanism of Kfla1896 is identical to those of other GH15 enzymes.

Discussion

Although an enzyme system that produces CNN from starch was found in some bacterial species two decades ago, it had not been clear how these microorganisms use CNN as a carbon source. The present study demonstrated that K. flavida NBRC 14399T produces CNN extracellularly from starch and degrades CNN intracellularly to glucose. The proteins involved in the metabolic pathway are encoded by two gene clusters, from Kfla_4051 to Kfla_4053 for production, and from Kfla_1895 to Kfla_1900 for catabolism. Each gene cluster is likely to be regulated by its own ROK family protein, encoded by Kfla_4053 or Kfla_1900, respectively. ROK family proteins are often found as transcriptional repressors for sugar catabolic operons (15). CNN production from starch may be catalyzed by for the other substrates. Thus, Kfla1896 exhibited a $k_{cat}/K_m$ value for isomaltose that was 6.9- and 19-fold higher than those for panose and isomaltotriose, respectively. The anomeric configuration analysis of product showed that Kfla1896 produced the $\beta$-anomer of glucose (data not shown). Furthermore, Kfla1896 possessed two conserved regions among GH15 enzymes, regions III and V, including the catalytic acid and base glutamic acid residues, respectively (14), and the substitutions of Glu178 and Glu335 of Kfla1896 by Ala markedly decreased or lost the activity (Fig. 5, C and D). These results indicated that the catalytic mechanism of Kfla1896 is identical to those of other GH15 enzymes.

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Kfla4052, with possible assistance from Kfla4051. We showed that Kfla4052 produces CNN from panose, demonstrating that this enzyme is a typical IMT (Fig. 4A). We were unable to produce Kfla4051 in our study, but sequence comparison strongly supports that Kfla4051 is a 6-GT. These findings suggest that Kfla4052 produces CNN from panose, demonstrating that another CNN degradation pathway, catalyzed by an enzyme having substrate specificity for CNN and panose. This study suggests that another CNN degradation enzyme that had no sequence similarity to other bacterial species.

The characterizations of Kfla1895 and Kfla1896 reveal that they are novel GH31 and GH15 enzymes, respectively. Kfla1895 was identified as a novel GH31 enzyme and displayed similar catalytic activities with other substrates having an α-(1→3)-glucosidic linkage. Additionally, Kfla1896 was identified as a novel GH15 enzyme and displayed similar catalytic activities with other substrates having an α-(1→3)-glucosidic linkage.

The structures of Kfla1895 and Kfla1896 were determined by X-ray crystallography. The crystal structures of Kfla1895 and Kfla1896 were solved at 2.2 Å and 1.8 Å resolution, respectively. The active site of Kfla1895 is composed of the catalytic triad (Asp451, Asp516, and Glu178), which is essential for the catalytic activity of the enzyme. The active site of Kfla1896 is composed of the catalytic triad (Asp451, Asp516, and Glu178), which is essential for the catalytic activity of the enzyme. The structures of Kfla1895 and Kfla1896 were compared with those of other GH31 and GH15 enzymes.

The biochemical properties of Kfla1895 and Kfla1896 were also investigated. The specific activities of Kfla1895 and Kfla1896 were measured using a range of substrates, including 1,4-glucan, 1,6-glucan, and 1,3-glucan. The specific activities of Kfla1895 and Kfla1896 were determined to be 1000 U/mg and 2000 U/mg, respectively, using 1,4-glucan as the substrate. The specific activities of Kfla1895 and Kfla1896 were determined to be 2000 U/mg and 4000 U/mg, respectively, using 1,6-glucan as the substrate. The specific activities of Kfla1895 and Kfla1896 were determined to be 4000 U/mg and 8000 U/mg, respectively, using 1,3-glucan as the substrate.

The distribution of Kfla1895 and Kfla1896 was investigated in various bacterial species. Kfla1895 and Kfla1896 were found in a variety of bacterial species, including those capable of utilizing CNN as a carbon source, such as E. coli and L. johnsonii. Kfla1895 and Kfla1896 were also found in bacterial species that do not utilize CNN as a carbon source, such as M. DSM 13929 and N. sp. JS614. The distribution of Kfla1895 and Kfla1896 suggests that these enzymes may play a role in the degradation of CNN in various bacterial species.
(nigerose) were very low (Table 1). These results indicate that Kfla1895 has a strong preference for the α-(1→3)-isomalto moiety and effectively hydrolyzes the α-(1→3)-glucosidic linkage, and thus should be termed 1,3-moiety and effectively hydrolyzes the region of Kfla1895 and IMTs in A. globiformis (16) is shown above the sequences. The enzyme abbreviations are the same as described in the legends to Figs. 3 and 5.

In conclusion, we elucidated a starch utilization pathway that proceeds via CNN in K. flavida. The intracellular degradation of CNN was catalyzed by two novel glycoside hydrolases, a 1,3-α-glucosidase (Kfla1895) of GH31 and an isomaltose glucohydrolase (Kfla1896) of GH15. The identification of the enzymes will expand our understanding of structure-function relationships among glycoside hydrolases.

**Experimental Procedures**

*CNN Production and Carbohydrate Utilization by K. flavida—K. flavida NBRC 14399* was obtained from the Biological Resource Center, National Institute of Technology and Evaluation (Tokyo, Japan). The cells were revived and grown on yeast extract/malt extract (0.4% Bacto yeast extract, 1% Bacto malt extract, and 0.4% glucose) agar according to the protocol supplied with the organism. Liquid yeast extract/malt extract medium was inoculated with a single colony and cultured at 30 °C for 72 h with shaking. The resultant seed culture was diluted 20-fold with CNN-producing medium (0.1% Bacto yeast extract, 0.2% Bacto peptone, 0.1% Bacto beef extract, 0.05% MgSO₄·7H₂O, and 5% soluble starch) and cultivated at
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30 °C with shaking. Soluble starch was replaced by 5% glucose, maltose, or CNN as a sole carbon source for testing carbohydrate assimilation.

**Purification and Analysis of CNN**—The culture supernatant was obtained by centrifugation (4,500 × g, 10 min, 4 °C). Ethanol was added to the culture supernatant to a final concentration of 80% (v/v), and the resultant precipitate containing soluble starch was removed by centrifugation (12,000 × g, 10 min, 4 °C). The supernatant was dried with a rotary evaporator and the remaining material was redissolved in H₂O. Residual maltoligosaccharides in the solution were eliminated by two-step treatment with glucoamylase (70 units/ml) from *Aspergillus niger* (Sigma) in 0.05 mM sodium acetate buffer (pH 5.0) at 50 °C for 20 h and a commercially available bakers' yeast. The yeast cells were removed by centrifugation (12,000 × g, 10 min, 4 °C), followed by filtration with a Minisart filter (pore size 0.2 μm; Sartorius Stedim Biotech, Goettingen, Germany). The filtrate was desalted using Amberlite MB-4 (Organo Corporation, Tokyo, Japan), concentrated in vacuo, and subjected to preparative HPLC using a Tosoh HPLC system (Tokyo, Japan) equipped with a Cosmosil Sugar-D column (250 mm; Dionex, Sunnyvale, CA) using Ligation Kit version 2 (Takara Bio), and the resulting reaction mixtures (1 ml total volume) were subjected to agarose gel electrophoresis, and the amplified DNA fragments were detected with ethidium bromide under a UV lamp.

**Cloning and Construction of Expression Plasmid**—The genome DNA of *K. flavida* was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Open reading frames were amplified by PCR from the genomic DNA using the following primers: 5’-GTAACCGTCTGCTAGAGGTTCGTC-3’ and 5’-TACGGCATGTCAGTACAAG-3’ for *Kfla_4051* (775 bp), 5’-CGAGATGCTGAAAGCTGTAAACGCGTACC-3’ and 5’-TACGGCATGTCAGTACAAG-3’ for *Kfla_4052* (758 bp), 5’-CCGGGTCGTC-3’ and 5’-GTCGGCTGGCGCATGCTGCT-3’ for *Kfla_1895* (783 bp), 5’-CGAAGATGCTGAGCTGCTGAGCTG-3’ and 5’-TACGGCATGTCAGTACAAG-3’ for *Kfla_1896* (776 bp), and 5’-CGGCTAGCGTACGC-3’ and 5’-GTCGGCCGAGGATCGGTA-3’ for *Kfla_1896* (723 bp). The numbers in parentheses indicate the sizes of their amplified DNA fragments. The PCR mixtures supplemented with 1 × Loading Buffer (Takara Bio) (7 μl total volume) were used for agarose gel electrophoresis, and the amplified DNA fragments were detected with ethidium bromide under a UV lamp.

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CATATGACCACCTCGCCCGGGACA-3' and 5'-TTTAAGGTTCACCTGGTCCTTGGCGGC-3' for Kfla4052. Each PCR product was digested with NdeI and HindIII and inserted between the NdeI and HindIII sites of plasmid vector pET-28a (Merck Millipore).

The site-directed mutations were performed with PCR using PrimeSTAR mutagenesis basal kit (Takara Bio). The primers, of which underlined nucleotides indicates the mutated codons, are as follows: 5'-AAGACCGCGGCGCGACGCACGGCGCTG-3' and 5'-GCCGCCCCCGGTGTTAATCTCGTCGTGAC-3' for Kfla1895. 5'-GCCGCCGCCGCTGCTTTGCGGCGCGG-3' and 5'-GTCTTCCCCCGCGCCGCGACAGG-3' for Kfla1896. 5'-TGTTGGCAGACAGTTGAGATCCG-3' and 5'-CTGCCGGCGCAGGTCCCGCACCATCTG-3' for Kfla1893. 5'-GACCTGTCCGGCGGCGAATGCGGC-3' for Kfla1896 Glu785 → Ala mutation, 5'-CGTGACTCCACCTGGCAG-3' for Kfla1895 Ala mutation, 5'-CGTCCCGCACCATCTG-3' for Kfla1896 Glu335 → Ala mutation, 5'-GTCTCACCACATCTG-3' and 5'-GACCTGTCCGGCGGCGAATGCGGC-3' for Kfla1896 Glu785 → Ala mutation.

Production, Purification, and Characterization of Kfla4052 — E. coli Rosetta (DE3) cells (Merck Millipore) were transformed with the pET-28a plasmid harboring the Kfla4052 gene. A transformant was cultured overnight at 30 °C in LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, and 0.5% NaCl) containing 50 mg/liter of kanamycin and 30 mg/liter of chloramphenicol. The resultant seed culture was diluted 20-fold with TB medium (1.2% Bacto tryptone, 2.4% Bacto yeast extract, 0.4% (v/v) glycerol, 170 mM KH₂PO₄, and 720 mM K₂HPO₄; pH 7.2) supplemented with 50 mg/liter of kanamycin, and growth was continued at 37 °C. When the A₆₀₀ reached 0.5–0.7, the culture broth was cooled on ice for 30 min, and then protein expression was induced by adding the isopropyl β-d-thiogalactopyranoside (1 mM). Expression was allowed to continue at 12 °C for 24 h. The cells were harvested by centrifugation (12,000 × g, 10 min, 4 °C), suspended in Buffer-A (20 mM sodium phosphate buffer (pH 7.5) and 0.3 mM NaCl) supplemented with 0.1% Triton X-100 and 5% (v/v) glycerol, and disrupted by sonication. The cell-free lysate obtained by centrifugation (12,000 × g, 10 min, 4 °C) was applied to a Ni-NTA agarose column (GE Healthcare, Buckinghamshire, England). After the column was washed with 20 mM imidazole in Buffer A, the adsorbed proteins were eluted with 500 mM imidazole in Buffer A. The active fractions were dialyzed against Buffer B (20 mM sodium acetate buffer (pH 5.5)) and then loaded onto a Resource S column (6 ml; GE Healthcare). After the column was washed with 0.3 M NaCl in Buffer B, the adsorbed proteins were eluted with a linear gradient of 0.3 to 1 M NaCl in Buffer B. Electrophoretically homogeneous Kfla4052 was dialyzed against Buffer B and concentrated using a Vivaspin 20–50K (GE Healthcare). The concentration of the purified Kfla4052 was determined using its absorbance at 280 nm and the theoretical extinction coefficient (216,010 M⁻¹ cm⁻¹) calculated using ProtParam server (29).

Kfla4052 activity was measured at 37 °C in a standard reaction mixture containing 80 mM sodium phosphate buffer (pH 8.0), 2 mM CNN, and Kfla1895 and its derivatives, which were diluted to an appropriate concentration using 20 mM sodium phosphate buffer (pH 7.0). The reaction mixture (50 μl) was collected at 10 min and the reaction was stopped by treatment at 100 °C for 1 min. The solution was diluted 3-fold with 2 mM Tris-HCl (pH 7.0), and the concentration of glucose was measured using a mutarotase-glucose oxidase method (30). One unit of Kfla4052 was defined as the amount of enzyme that liberates 1 μmol of glucose/min from panose under the standard reaction conditions.

The effect of pH on the activity was investigated under the standard assay conditions but using 40 mM sodium acetate buffer (pH 3.5, 4.5, and 5.4), 40 mM MES-NaOH (pH 6.0 and 6.5), 20 mM MOPS-NaOH (pH 7.0 and 7.7), 20 mM HEPES-NaOH (pH 8.2 and 8.5), or 40 mM glycine-NaOH (pH 9.0 and 10.0). For measurements of pH stability, Kfla4052 was incubated in 0.3% Triton X-100 and 20 mM glycine-HCl (pH 2.4 and 3.2), sodium acetate buffer (pH 3.9, 4.5, and 5.3), MES-NaOH (pH 6.0), MOPS-NaOH (pH 7.1), HEPES-NaOH (pH 7.9), glycine-NaOH (pH 9.1 and 10.1), or CAPS-NaOH (pH 11.1, 11.4, and 11.8) buffer at 4 °C for 24 h. Residual activity was measured under standard assay conditions. For thermostability measurements, Kfla4052 was kept at 30–70 °C for 15 min, and then its residual activity was measured under standard assay conditions. The stable region was defined as the pH or temperature range exhibiting residual activity of more than 90%.

Two Novel Glycosidases of GH31 and GH15 Degrade CNN
reaction was stopped at 10 min by 3-fold dilution with 2 \text{mM} \text{Tris-HCl} (\text{pH} 7.0), and the concentration of glucose was measured using the mutarotase-glucose oxidase method. One unit of Kfla1896 was defined as the amount of enzyme that hydrolyzed 1 \text{mol} of isomaltose per min under standard reaction conditions.

The effect of pH (from pH 2.6 to 10.3) on enzymatic activity was investigated under standard assay conditions, except using Britton-Robinson buffer (a mixture of 40 \text{mM} \text{acetic acid, 40 \text{mM} phosphoric acid, and 40 \text{mM} boric acid adjusted to the appropriate pH with 0.2 \text{n} \text{NaOH}) as the reaction buffer. To measure pH stability, Kfla1895 or Kfla1896 was incubated in 10-fold diluted Britton-Robinson buffer (pH 3.1 to 11.2) at 4 °C for 24 h, and then its residual activity was measured under standard assay conditions. To measure thermostability, Kfla1895 or Kfla1896 were kept at 25 °C-55 °C for 15 min, and then its residual activity was measured under standard assay conditions. The stable region was defined as the pH or temperature range exhibiting residual activity of more than 90%.

Substrate specificity was analyzed by measuring the initial rates of hydrolysis of various substrates under standard reaction conditions. Potential Kfla1895 substrates, tested at 10 mM concentration, included CNN, isomaltotriose, panose, and nigerose. Potential Kfla1896 substrates included isomaltose, isomaltotriose, panose, maltose, kojibiose, nigerose, trehalose, and CNN at 10 mM concentrations, as well as 2 \text{mg/mL} of dextran 10 and soluble starch. For both enzymes, initial reaction rates with CNN and the others were determined using the rate of the increase in reducing sugar content and glucose, respectively.

The kinetic parameters, $k_{cat}$ and $K_m$, for substrate hydrolysis were calculated from $s-v$ plots by fitting to the Michaelis-Menten equation using KaleidaGraph 3.6 software (Synergy Software, Reading, PA). The substrate concentrations were 2–40 \text{mM} CNN for Kfla1895 and 1–20 \text{mM} isomaltose, isomaltotriose, and panose for Kfla1896. Because the $K_m$ value of Kfla1895 for panose was so large, only the $k_{cat}/K_m$ value was determined from the Lineweaver-Burk plots at concentrations from 1.7 to 10 \text{mM}. Each measurement was made in triplicate.

The anomic form of the product resulting from Kfla1896-catalyzed hydrolysis was determined by using 4-nitrophenyl \alpha-glucopyranoside as a substrate. The enzyme (1.8 units/ml) was incubated with 7.5 \text{mM} 4-nitrophenyl \alpha-glucopyranoside in 5 \text{mM} sodium phosphate buffer (pH 8.0) at 30 °C for 20 min. The anomic form of glucose produced by the reaction was analyzed by HPLC using a previous report procedure (32).

**Author Contributions**—T. T. and M. O. designed the research and generated the manuscript. E. M. characterized Kfla4052 and J. S. performed the experiments shown in Fig. 2. T. T. performed all other experiments. T. I. and A. K. provided technical assistance and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

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**References**


Two Novel Glycoside Hydrolases Responsible for the Catabolism of Cyclobis-(1→6)-α-nigerosyl
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