Construction of Chimeric $\beta$-Glucosidases with Improved Enzymatic Properties*

(Received for publication, February 6, 1995, and in revised form, July 6, 1995)

Ajay Singh and Kiyoshi Hayashi‡

From the Biomaterials Conversion Laboratory, National Food Research Institute, Tsukuba, Ibaraki 305, Japan

The amino acid sequences of $\beta$-glucosidases from Cellvibrio gilvus and Agrobacterium tumefaciens show about 40% similarity. The pH/temperature optima and stability and substrate specificities of the two enzymes are quite different. $C$. gilvus $\beta$-glucosidase exhibits an optimum pH of 6.2-6.4 and temperature of 35°C, whereas the corresponding values for $A$. tumefaciens are 7.2-7.4 and 60°C, respectively. The substrate specificity of $A$. tumefaciens enzyme toward different aryl glycosides is broader than $C$. gilvus enzyme. To analyze these properties further, three chimeric $\beta$-glucosidases were constructed by substituting segments from the C-terminal homologous region of $C$. gilvus $\beta$-glucosidase gene with that of $A$. tumefaciens. The chimeric enzymes were characterized with respect to pH/temperature activity and stability and substrate specificity. Chimeric enzymes exhibited chromatographic behavior similar to that of $C$. gilvus enzyme. However, enzymatic properties of chimeras were admixtures of those of the two parents.

The chimeric enzymes were optimally active at 45-50°C and pH 6.6-7.0. $K_m$ values of chimeric enzymes for the various saccharides were admixtures of both parental enzymes. These results suggest that the two domains of $C$. gilvus and $A$. tumefaciens enzymes probably can fold independently. The homologous C-terminal region in $\beta$-glucosidase appears to play an important role in determining enzyme characteristics. Changes in the properties on substitution of segments in this region might be related to the enzyme specificity, and $\beta$-glucosidases with improved properties can be prepared by manipulating this region.

The enzyme $\beta$-glucosidase (EC 3.2.1.21) catalyzes the hydrolysis of alkyl- and aryl-$\beta$-D-glucosides (methyl-$\beta$-D-glucoside and p-nitrophenyl-$\beta$-D-glucoside) as well as glycosides containing only carbohydrate residues (Cellulase). On the basis of substrate specificity, $\beta$-glucosidases can be classified as aryl-$\beta$-glucosidases, cellobiases, and those hydrolyzing both aryl-$\beta$-glucosides and oligosaccharides. The last group is often found in cellulytic microorganisms (1, 2). On the basis of sequence homology, $\beta$-glucosidases have been divided into two subfamilies (2). BGA ($\beta$-glucosidases and phospha-$\beta$-glucosidases from bacteria to mammals) and BGB ($\beta$-glucosidases from yeasts, molds, and rumen bacteria). It is one of the components of the cellulase enzyme complex required for the hydrolysis of cellulose to glucose by catalyzing the final step which converts cellobiose to glucose (3, 4).

The study of these enzymes has been facilitated by the use of recombinant DNA technology (1, 5, 6). Although a number of cellulase genes including several $\beta$-glucosidases have been cloned and expressed in both Escherichia coli and Saccharomyces cerevisiae (7-10), their enzymological properties, especially structure-function relationships, have not been well understood, partially because most of the cellulases show little sequence homology. Analysis of structure-function relationships may be facilitated by the formation of chimeric genes/ enzymes produced by gene fusion (11).

Cellvibrio gilvus, a cellulose-metabolizing bacterium, has the unique property of producing cellobiose in high yields from acid-swollen cellulose (12). The isolation and characterization of the cellulase, xylanase, and $\beta$-glucosidase systems of this organism (13-15) as well as the cloning, analysis, and manipulation of the genes coding these enzymes (16, 17) have been investigated in our laboratory. The $\beta$-glucosidases from $C$. gilvus share conserved regions in $\beta$-glucosidases from different organisms. The nucleotide sequence of the $\beta$-glucosidase gene revealed that this enzyme belongs to the BGB group of $\beta$-glucosidases (15). The amino acid sequences of the $C$. gilvus $\beta$-glucosidase gene show significant similarity (about 40%) with those of a $\beta$-glucosidase gene from Agrobacterium tumefaciens (18). Despite this similarity, their enzymatic properties, especially pH activity, thermal stability, and substrate specificity, are quite different. To analyze these properties further, chimeric $\beta$-glucosidases were constructed between them by substituting different segments from one enzyme in the C-terminal homologous region of the other and comparing the enzyme characteristics of parental and chimeric enzymes. The C-terminal region seems to be important for $\beta$-glucosidase activity, since deletion of more than a 70-base pair fragment from the C-terminal part of $C$. gilvus $\beta$-glucosidase gene resulted in the loss of enzyme activity.1 Although, the deletion of about 100 amino acid residues near the C-terminal region of the $\alpha$-amylase gene did not affect enzyme activity (19), cyclomaltdextrin glucanotransferases lacking 30 amino acids (20) and an endoglucanase lacking 75 amino acids (21) from the C-terminal end showed no enzyme activity. Keeping in mind the importance of the C-terminal region and the estimated location of the catalytic center of Asp-291 in the N-terminal region of $C$. gilvus (15), the C-terminal region was selected for the construction of chimeric enzymes.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli NM522 hsdS2 F lacZMd15 (lac-pro) (F pro lacZMD15) and E. coli DH5α F- glyD lacZMD15 recA1 and A1 gyrA thi-1 hsdR17 (Msr dim) supE44 relA (lacY1-argF) U169 were hosts for $\beta$-glucosidase genes from $C$. gilvus and $A$. tumefaciens, respectively. pCG5 and pCG6 were the recombinant plasmids.

1 T. T. Hoa and K. Hayashi, unpublished observations.

* This work was supported by Ministry of Agriculture, Forestry, and Fisheries Grant BMP-95-V-4-S and by the Japan International Science and Technology Exchange Center/Research Development Corporation of Japan (to A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 81-298-38-8071; Fax: 81-298-38-7996.
Chimeric β-Glucosidases

Carrying C. glivus (15, 16) and A. tumefaciens (18) β-glucosidase genes, respectively.

DNA Manipulation—Recombinant DNA techniques and methods for agrose gel electrophoresis were followed as described by Sambrook et al. (22). Plasmid DNA was prepared by alkaline lysis method. The digestion by restriction enzymes was carried out in the appropriate buffer at concentrations of 3-10 units per μg of DNA at appropriate temperatures. The completion of the reaction was confirmed by agarose gel electrophoresis. Sephaglas BandPrep kit (Pharmacia LKB Biotechnology Inc.) was used for the extraction and purification of DNA from agarose gels.

Construction of Chimeras—Plasmid pCG5 was used as a vector. Three restriction sites, the BstBI and BsaBI sites in the C-terminal homologous region of pCG5 were used to construct three chimeric β-glucosidase genes. Plasmid pCG5 was digested with BsmI followed by blunting and dephosphorylation before ligation with insert DNA. A 1657-base pair fragment was obtained by digestion of pcgB1 with NdeI and Hinfl. This fragment was recovered from agarose gel, purified, and blunted. Plasmid pCHB1SM1 was constructed by ligation of this fragment with the BsmI-digested fragment of pCG5. Plasmid pCHAGE1 was constructed by ligation of an AgeI-digested fragment of pCG5 and a 1670-base pair AvaI fragment of pcgB1. Plasmid pHCH-SAB1 was constructed by ligation of a BsaBI-digested fragment of pCG5 and a 1227-base pair SfiI-Hinfl fragment of pcgB1. The clones producing β-glucosidase were detected by fluorescence from 4-methylumbelliferone of a UV transilluminator after growing on LB agar plates containing 1 mM 4-methylumbelliferone-β-glucoside.

Production and Purification of β-Glucosidases—Cells were grown overnight in 5 ml of Luria-Bertani (LB) medium at 30°C. One milliliter of culture was used to inoculate 1 liter of LB medium supplemented with 50 μg/ml ampicillin. Cultures were incubated aerobically, and cells were harvested in the late log phase of the growth by centrifugation. The cells were suspended in 25 ml MOPS2 buffer (pH 6.5) and sonicated using a Branson Sonifier Model 250/450. Cell debris was separated by centrifugation, and the supernatant was obtained as a crude enzyme preparation.

C. glivus, A. tumefaciens, and chimeric β-glucosidase preparations were partially purified by ion exchange chromatography. C. glivus and the chimeric enzyme preparations were applied to a FPLC system (Pharmacia LKB Biotechnology Inc.) using a column of SP Sepharose Fast Flow HiLoad™ 26/10 (bed volume 53-58 ml) equilibrated with 25 mM acetate buffer (pH 5.0). The proteins were eluted with a linear gradient of 0–1 M NaCl in the same buffer. In the case of β-glucosidases of A. tumefaciens, the enzyme preparation was applied to a column of Q Sepharose Fast Flow HiLoad™ 26/10 (bed volume 53-58 ml) equilibrated with 20 mM bis-tris propane (pH 6.5). The proteins were eluted with a linear gradient of 0–1 M NaCl in the same buffer. The partially purified fractions were used for the determination of enzyme characteristics.

For determination of kinetic parameters, C. glivus β-glucosidase and chimeric CHBSM β-glucosidase were further purified on a large scale. Ten liters of cultures were centrifuged at 5000 × g for 10 min, and cells were suspended in 100 ml of 25 mM MOPS buffer (pH 6.5). The enzyme solution was obtained after sonication of the cells and removal of the cell debris by centrifugation. The enzyme solution was applied to a column of SP Sepharose Fast Flow. The enzymes were eluted with a linear gradient of 0–1 M NaCl in 20 mM acetate buffer, pH 5.0. Active fractions were pooled, dialyzed, and applied to a column of Mono Q (Pharmacia). The enzymes were eluted with a linear gradient of 0–1 M NaCl. The active fractions were pooled, concentrated, and finally applied to a gel filtration column of Superose 6 (Pharmacia). The enzymes were eluted with 25 mM acetate buffer (pH 5.0) containing 0.15 M NaCl. Homogeneity of the purified enzyme preparations was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a PhastSystem (Pharmacia).

β-Glucosidase Assay—In the standard assay, an aliquot of suitably diluted enzyme solution was incubated in the reaction mixture containing 25 mM MOPS buffer (pH 6.5) and 2 mM p-nitrophenyl-β-glucoside (pNPG) for 30 min at 30°C. The reaction was stopped by the addition of 0.2 M glycine-NaOH (pH 10.5), and the amount of p-nitrophenol released was measured by the absorbance at 405 nm. One unit of β-glucosidase activity was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per min under the above conditions.

Other Methods—Kinetic parameters were determined by the double reciprocal plot method of Lineweaver and Burk (23) using p-nitrophenyl derivatives of saccharides dissolved at various concentrations in 25 mM MOPS buffer, pH 6.5, as suitably diluted enzyme solution. The reaction mixture was incubated at 30°C for 10 min and then boiled for 5 min to terminate the enzyme reaction. Released glucose was measured by a glucose oxidase-peroxidase kit, Glucose CII Test, Wako Chemical Industries. Products of enzymatic action on cello-oligosaccharides were analyzed by the HPLC system (DIONEX) equipped with a DIONEX pulsed amperometric detector. The prepacked column was a DIONEX Carboxypak™ PA1 (4 × 250 mm). Solvents A (100 mM NaOH) and B (100 mM NaOH, NaOAc) were used as eluents (flow rate 1 ml/min) at 92% and 8% concentrations, respectively. Sugars were identified and quantified by comparison with retention times of authentic saccharide standards.

Enzymes and Chemicals—All the restriction enzymes (TaKaRa and BioLabs), DNA ligation kit (TaKaRa), and Sephaglas BandPrep DNA extraction and purification kit (Pharmacia) used in this study were purchased from commercial sources according to the manufacturer’s recommendations. 4-Methylumbelliferyl-β-D-glucoside and aryl-glycosides were obtained from Sigma and cello-oligosaccharides from Seikagaku Kogyo. All other chemicals used were reagent grade.

RESULTS AND DISCUSSION

Construction of Chimeric β-Glucosidases—β-Glucosidase genes of C. glivus (15, 16) and A. tumefaciens (18) have been cloned previously, and their nucleotide sequences have been reported. Despite the significant similarity of the deduced amino acid sequences, most of the enzymatic features of the two β-glucosidases were quite distinct. β-Glucosidase from A. tumefaciens shows higher pH and temperature optimas as well as broader substrate specificity as compared to the one from C. glivus. To analyze these properties further as well as to study the effect of the shuffling of homologous segments in the C-terminal region, three chimeric β-glucosidases derived from two genes were constructed.

The amino acid sequences of β-glucosidases from C. glivus and A. tumefaciens show significant similarity on most of the parts. In particular, the region from Ala-472 to Pro-741 of the C. glivus β-glucosidase gene is shown in Fig. 1. Considering the translation frame and similar regions of both genes, three enyzmes in the β-glucosidase gene (plasmid pCG5) were selected for substitution with the A. tumefaciens β-glucosidase gene. Schematic representation of the structure of the chimeric β-glucosidase gene is shown in Fig. 2. The region between the NdeI and Hinfl sites starting from Cys-517 and over stop codon in pcgB1 were substituted at the BsmI site of pCG5 to obtain the chimeric enzyme CHBSM. Similarly, two other chimeric enzymes were obtained by substituting the region between the two AvaI sites starting from Ala-594 in pcgB1 at the AgeI site of pCG5 (CHAGE) and the region between SflI and Hinfl sites starting from Asp-660 in pcgB1 at the BsaBI site of pCG5 (CHBSA). The clones expressing chimeric enzymes were purified, and their plasmids were characterized by restriction analysis. Plasmid pCHBSM encoding chimeric enzyme CHBSM has two SflI sites in the inserted fragment of pcgB1, whereas pCHAGE1 and pCHBSAS1 encoding chimeras CHAGE and CHBSA, respectively, have two EcoRV sites in the inserted fragments of pcgB1 (Fig. 3).

Characteristics of Chimeric β-Glucosidases—Parental and chimeric β-glucosidases were partially purified by the FPLC system using either SP Sepharose (cation exchange) or Q Sepharose (anion exchange) columns in order to determine.
enzymatic properties. Marked differences in the behavior of \(\beta\)-glucosidases from \(C.\) gilvus and \(A.\) tumefaciens have been observed during the course of purification using ion exchange chromatography. The cation exchange column was used at pH 5.0 to elute \(\beta\)-glucosidases from \(C.\) gilvus, whereas the anion exchange column successfully eluted \(\beta\)-glucosidases from \(A.\) tumefaciens at pH 6.5. Chimeric \(\beta\)-glucosidasesshowing behavior similar to that of \(\beta\)-glucosidase from \(C.\) gilvus in ion exchange chromatography were purified about 40-fold using an SP Sepharose column.

The pH optima for \(C.\) gilvus and \(A.\) tumefaciens enzymes are 6.2–6.4 and 7.2–7.4, respectively. These enzymes also show marked differences in their temperature optima. \(\beta\)-Glucosidase from \(C.\) gilvus is optimally active at 35°C, whereas that of \(A.\) tumefaciens exhibits maximum activity at 60°C. With regard to

FIG. 1. Homology in amino acid sequences of \(\beta\)-glucosidases from \(C.\) gilvus and \(A.\) tumefaciens. AT and CG represent \(A.\) tumefaciens and \(C.\) gilvus, respectively. A, schematic representation of sequence homology in N-terminal (solid) and C-terminal (hatched) regions of \(\beta\)-glucosidase genes. B, amino acid sequences of \(A.\) tumefaciens and \(C.\) gilvus \(\beta\)-glucosidases in the C-terminal region. Identical and similar amino acid residues are designated by * and ., respectively. Chimeric enzymes were constructed by shuffling the regions marked by arrowheads.

FIG. 2. Schematic representation of parental and chimeric \(\beta\)-glucosidase genes. Light and dark bars represent regions derived from \(C.\) gilvus and \(A.\) tumefaciens, respectively. Restriction enzymes used for the construction of chimeric enzymes are shown with open arrowheads, whereas the restriction enzymes used for the confirmation of chimeric plasmids are shown with filled arrowheads.

FIG. 3. Restriction analysis of chimeric plasmids on agarose gel electrophoresis. Lane 1, HindIII digest of \(\lambda\)DNA marker; lane 2, BioMarker; lane 3, SfiI-digested pCHBSM1; lane 4, BamHI/EcoRV-digested pCHAGE1; lane 5, BamHI-digested pCHBSAB1; lane 6, SfiI-digested pCG5; and lane 7, BamHI-digested pCG5. The 1.2-kilobase band in pCHBSM1 and 0.7-kilobase band in pCHAGE1 and pCHBSAB1 were created by the SfiI and EcoRV sites, respectively, in the inserted gene.
to heat stability, β-glucosidase from C. gilvus shows complete activity up to 30°C, retains about 80% of its maximum activity at 35°C, and inactivates completely at 55°C. On the other hand, A. tumefaciens enzyme is stable up to 55°C, and, even at 65°C, it retains 60% of its maximum activity. A. tumefaciens enzyme specificity toward aryl-glycoside substrates is broader than that of C. gilvus enzyme.

The pH activity profilesof chimeric β-glucosidases are shown in Fig. 4. Chimeric enzymes showed intermediate profiles of their parents. CHBSM enzyme exhibited the maximum activity at pH 6.6–7.0, about 40% at pH 8.0, and no activity at pH 10.0. The optimum pH of CHAGE enzyme was 6.8–7.0 with about 35% activity at pH 8.0. CHBSA enzyme was optimally active at pH 6.6 and inactivated at pH 9.0. All the chimeras were stable between pH 4 and 9, whereas the β-glucosidases from C. gilvus and A. tumefaciens were stable at pH 4–8 and pH 5–10, respectively. Substitution of segments in the homologous C-terminal region seems to have a marked influence on pH activity and stability. In Bacillus cyclomaltodextrin glucanotransferase (20) and celulase (24), pH activity profiles were found to be influenced by the N- and the C-terminal parts.

The chimeric β-glucosidases also exhibited a significant variation in temperature optimum from their parent enzymes (Fig. 5). The chimeric enzymes were optimally active at 45–50°C, showing an intermediate temperature optimum between C. gilvus and A. tumefaciens enzymes. CHBSM exhibited maximum activity at 50°C, and 61% of its maximum activity at 60°C. On the other hand, CHAGE was optimally active at 50°C and exhibited 52% of its maximum activity at 60°C. CHBSA showed the temperature optima of 45°C with no activity at 70°C. Heat stability experiments revealed that CHBSM enzyme was completely active up to 45°C, retained about 65% of its maximum activity at 55°C, and was completely inactivated at 55°C. CHAGE enzyme was stable up to 40°C, and, even at 55°C, 50% of its maximum activity was retained. CHBSA was least stable among the three chimeras. It was stable up to 40°C, and retained only 20% of its maximum activity at 55°C. The enzymes at which 50% loss of the enzyme activity occurred were 41, 67, 57, 55, and 50°C for C. gilvus, A. tumefaciens, CHBSM, CHAGE, and CHBSA enzymes, respectively. Thus heat stability of chimeric enzymes was increased by 9–16°C as compared to C. gilvus enzyme.

Heat stability may be influenced by only a few amino acid substitutions (17, 25). In general, protein stability increases with the insertion into an α-helix of helix-forming amino acids (alanine, glutamic acid etc.) and decreases with the insertion of helix-breaking amino acids (proline, glycine etc.). The secondary structures of the parental and chimeric enzymes were predicted by Robson’s method (26). There were similar numbers of helix-breaking but more helix-forming amino acid residues in the α-helix regions of chimeric enzymes than C. gilvus enzyme, suggesting that it could be one of the factors influencing the heat stability of chimeras. Hydrophobic interaction inside the protein molecule is another important factor in stabilizing protein structure. Hydrophobic cluster analysis (27, 28) of native and chimeric enzymes revealed that the amino acid substitution from C. gilvus to A. tumefaciens significantly increased the hydrophobic properties of the chimeric enzymes. These substitutions might be important for heat stability of β-glucosidase. Thus, the pH activity and heat stability were changed dis-
 distinctly by substituting different segments of C. gilvus β-glucosidase gene with that of A. tumefaciens. It is interesting to note that these changes were more pronounced with the increased size of the insertion fragment. For example, CHBSM containing the largest insertion fragment from A. tumefaciens β-glucosidase exhibited broader pH optima than the other two chimeras. Thermal stability was also found to be in the order of CHBSM > CHAGE > ChBsa. In chimeric isopropylmalate dehydrogenase from an extremophile, Thermus thermophilus, and a mesophile, Bacillus stearothermophilus, the stability of each chimeric enzyme was approximately proportional to the content of the amino acid sequence from the T. thermophilus enzyme (29).

Substrate Specificity—The substrate specificity of the chimeric and parental enzymes was studied using various arylglycosides as shown in Table I. β-Glucosidase from C. gilvus has rather strict specificity toward glucose residues in arylglycosides. This enzyme hydrolyzed p-nitrophenyl-β-D-galactoside, and p-nitrophenyl-β-D-glucoside. AT and CG represent A. tumefaciens and C. gilvus, respectively.

Substrate Specificity—The substrate specificity of the chimeric and parental enzymes was studied using various arylglycosides as shown in Table I. β-Glucosidase from C. gilvus has rather strict specificity toward glucose residues in arylglycosides. This enzyme hydrolyzed p-nitrophenyl-β-D-galactoside, and p-nitrophenyl-β-D-glucoside. AT and CG represent A. tumefaciens and C. gilvus, respectively.

### Table I

**Kinetic parameters of parental enzymes and chimeric enzymes**

All the enzymes AT, BSM, AGE, BSA, and CG were inactive (less than 0.1% of the activity to pNPG) to the following substrates: p-nitrophenyl-β-D-mannoside, p-nitrophenyl-α-D-glucoside, and p-nitrophenyl-N-acetyl-β-D-galactosaminide. Only AT shows measurable activity (0.5% activity to that of pNPG) to p-nitrophenyl-N-acetyl-β-D-glucosaminide. AT and CG represent A. tumefaciens and C. gilvus, respectively.

| Substrate                  | AT     | CHBSM | CHAGE | CHBSA | CG     |
|----------------------------|--------|-------|-------|-------|--------|
| p-Nitrophenyl-β-D-glucoside|        |       |       |       |        |
| $K_m$ (mM)                 | 0.032  | 0.270 | 0.291 | 0.273 | 1.806  |
| $V_{max}$ (%)              | 100    | 100   | 100   | 100   | 100    |
| p-Nitrophenyl-β-D-xylgoside|        |       |       |       |        |
| $K_m$ (mM)                 | 0.005  | 0.004 | 0.004 | 0.005 | 6.261  |
| $V_{max}$ (%)              | 14     | 9.1   | 7.2   | 7.7   | 0.81   |
| p-Nitrophenyl-β-D-galactoside|      |       |       |       |        |
| $K_m$ (mM)                 | 13.1   | 16.6  | 15.1  | 34.4  | 10.8   |
| $V_{max}$ (%)              | 63     | 43    | 32    | 49    | 0.15   |
| p-Nitrophenyl-β-D-fuco side|        |       |       |       |        |
| $K_m$ (mM)                 | 0.123  | 0.210 | 0.166 | 0.277 |        |
| $V_{max}$ (%)              | 20     | 14    | 10    | 11    | <0.001 |

* Since partially purified enzymes were used in this assay, measured $V_{max}$ values were expressed as relative values to the $V_{max}$ values for pNPG.

### Table II

**Relative hydrolysis rates of cellobiose and related saccharides by parental and chimeric enzymes**

| Substrate (1 mM) | Relative activity (%) |
|-----------------|-----------------------|
|                 | AT        | CHBSM | CHAGE | CHBSA | CG    |
| 4-Methylumbelliferyl-β-D-glucoside | 100      | 100   | 100   | 100   | 100   |
| Celllobiose     | 20.2     | 20.3  | 11.2  | 16.0  | 11.9  |
| Celiotriase     | 33.9     | 40.5  | 29.9  | 37.1  | 34.5  |
| Celiotetraose   | 15.4     | 41.6  | 29.7  | 36.4  | 39.2  |
| Celiotetraose   | 12.1     | 41.9  | 32.1  | 36.8  | 41.7  |
| Cellhexitol     | 9.7      | 40.5  | 31.9  | 43.4  | 49.5  |

* Sufficient enzyme was added to cause a linear release of product during the first 10 min of reaction at pH 6.5 and 30°C. Activity was determined by measuring the release of glucose (glucose oxidase-peroxidase kit, Glucose CII Test, Wako). The relative rate of hydrolysis of an arylglycoside is expressed as a percentage of that obtained with 4-methylumbelliferyl-β-D-glucoside. AT and CG represent A. tumefaciens and C. gilvus, respectively.

chimeric enzymes (0.270 - 0.293 mM) were just between the two parental enzymes of C. gilvus (1.81 mM) and A. tumefaciens (0.032 mM). All enzymes including three chimeric enzymes strictly recognize the hydroxy residue at the C-2 position of β-glucose since p-nitrophenyl-β-D-mannoside was not hydrolyzed. These results suggest that in chimeric enzymes, the two domains of C. gilvus and A. tumefaciens β-glucosidases probably can be folded into active form correctly. The catalytic efficiency ($K_{cat}$) of one of the chimeric enzymes of CHBSM was measured to be $3 \times 10^3$ s⁻¹, and it is slightly lower than that of the C. gilvus enzyme, $8.9 \times 10^2$ s⁻¹. It might indicate that the sterostructure of the chimeric enzyme is to a certain extent perturbed in limited regions.

While the $K_m$ value of the chimeric citrate synthases similarly have been found to be lower than those of the parental enzymes (30), substrate affinity decreased by about 2-fold in active human-yeast chimeric phosphoglycerate kinase engineered by domain interchanges (31). However, no significant differences were found between the $K_m$ values of parental and chimeric isopropylmalate dehydrogenases (23). Replacement of the catalytic base Glu-400 by glutamine in Aspergillus niger glucoamylase was found to affect both substrate ground-state binding and transition state stabilization (32). $K_m$ values for maltose and maltolheptaose were 12- and 3-fold higher for the Glu-400 → Glu mutant, with $K_{cat}$ values 35- and 60-fold lower, respectively, as compared with those of the wild type enzyme. Similarly, in Aspergillus awamori glucoamylase mutants, Ser-119 → Tyr, Gly-183 → Lys, and Ser-184 → His, slightly higher activity for maltose hydrolysis and lower activity for isomaltose as compared with the wild type enzyme was observed by Siersk and Svensson (33). The observed increase in selectivity was attributed to the stabilization of the maltose transition-state complex for each enzyme. Modulation of binding energy by...
mutation could be attributed to modification in hydrogen bonding (32, 34, 35).

The relative rates of hydrolysis of cello-oligosaccharides by parental and chimeric enzymes are shown in Table I. 4-Methylumbelliferyl-β-glucoside was found to be the best substrate for all of the enzymes. Both C. glvis and A. tumefaciens β-glucosidases releases 4-methylumbelliferone and glucose in parallel from 4-methylumbelliferyl-β-glucoside (data not shown). C. glvis enzyme hydrolyzes cellobiose only 11.9% as fast as 4-methylumbelliferyl-β-glucoside, whereas hydrolysis rates of cellobiose by A. tumefaciens enzyme was 20.2%. Hydrolysis rates of cellotetraose, cellopentaose, and cellohexaose by C. glvis enzymes were 3–5 times higher than that of the A. tumefaciens enzyme. The hydrolysis rates of these oligosaccharides by chimeric enzymes were similar to that of the C. glvis enzyme and higher than that of the A. tumefaciens enzyme. The products released by enzymatic action on different cello-oligosaccharides were analyzed by HPLC. The C. glvis enzyme hydrolyzed each oligosaccharide into smaller oligosaccharides. However, cellobiose was hardly hydrolyzed. On the other hand, the A. tumefaciens enzyme hydrolyzed each oligosaccharide into glucose and the oligosaccharides smaller than the original ones by one glucose unit. The chimeric enzymes more or less exhibited patterns similar to that of C. glvis enzyme.

Genetic construction of chimeric enzymes from two functionally related proteins, sharing extensive sequence similarity, is expected not only to provide valuable information on the structure-function relationship of the parent proteins, but also to prepare enzymes with improved properties. Enzymatic activities are one of the sensitive criteria for judging the correct folding of engineered proteins. Our results demonstrate that different combinations of homologous C-terminal regions of β-glucosidases from C. glvis and A. tumefaciens resulted in the formation of enzymatically active chimeric species. The C-terminal region in the β-glucosidase gene plays an important role in determining enzyme characteristics, and the changes in enzymatic properties on substitution of the C-terminal segments might be related to enzyme specificity. Chimeric β-glucosidases with improved enzymatic properties can be prepared in a convenient and effective way by manipulating this region.

Acknowledgments—We are thankful to Drs. L. A. Castle, University of California, and R. O. Morris, University of Missouri, for useful discussions and for providing the recombinant plasmid-carrying β-glucosidase gene from A. tumefaciens. Thanks are also due to Drs. Y. Kashiwagi, H. Taniguchi, and S. Sasaki for critical discussion. Chika Aoyagi provided excellent technical assistance.

REFERENCES
1. Beguin, P. (1990) Annu. Rev. Microbiol. 44, 219–248
2. Paavilainen, S., Hellman, J., and Korpeila, T. (1993) Appl. Environ. Microbiol. 59, 927–932
3. Ryu, D. Y., and Mandels, M. (1980) Enzyme Microb. Technol. 2, 91–101
4. Shewale, J. G. (1982) Int. J. Biochem. 14, 433–443
5. Glick, B. R., and Pasternak, J. J. (1989) Biotechnol. Adv. 7, 363–386
6. Kuhad, R. C., and Singh, A. (1993) Crit. Rev. Biotechnol. 13, 151–172
7. Love, D. R., and Streiff, M. B. (1987) Biol Technology 5, 384–387
8. Bergs, P., Gilkes, N. R., Killburn, D. G., Miller, R. C., O’Neill, G., and Warren, R. A. J. (1987) Crit. Rev. Biotechnol. 9, 129–162
9. Wakarchuk, W. W., Greenberg, N. M., Killburn, D. G., Miller, R. C., Jr., and Warren, R. A. J. (1988) J. Bacteriol. 170, 301–307
10. Takano, M., Moriyama, R., and Ohmiya, K. (1992) J. Ferment. Bioeng. 73, 79–88
11. Wales, M. E., and Wild, J. R. (1993) Methods Enzymol. 202, 687–706
12. Starov, W. O., and King, K. W. (1960) J. Biol. Chem. 235, 303–307
13. Haga, K., Kitaoka, M., Sasaki, T., and Taniguchi, H. (1991) Agric. Biol. Chem. 55, 1959–1967
14. Kashiwagi, Y., Aoyagi, C., Sasaki, T., and Taniguchi, H. (1991) Agric. Biol. Chem. 55, 2553–2559
15. Kashiwagi, Y., Aoyagi, C., Sasaki, T., and Taniguchi, H. (1993) J. Ferment. Bioeng. 75, 159–165
16. Kashiwagi, K., Aoyagi, C., Sasaki, T., and Taniguchi, H. (1993) in Genetics, Biochemistry and Ecology of Lignocellulose Degradation (Shimada, K., Ohmiya, K., Kobayashi, Y., Hoshiba, S., Sakka K., and Karita, S., eds) pp. 368–377, UNI Publishers Co., Ltd., Tokyo
17. Singh, A., Hayashi, K., Hao, T. T., Kashiwagi, Y., and Tokuyasu, K. (1995) Biochem. J. 305, 715–719
18. Castle, L. A., Smith, K. D., and Morris, R. O. (1992) J. Bacteriol. 174, 1478–1486
19. Yamane, K., Hirata, Y., Furusato, T., Yamazaki, H., and Nakayama, A. (1984) J. Biochem. (Tokyo) 96, 1849–1858
20. Kurosaka, K., Song, K.-B., Hayashi, K., Kudo, T., and Horikoshi, K. (1989) J. Gen. Microbiol. 135, 3447–3457
21. Ohmiya, K., Deguchi, H., and Shimizu, S. (1991) J. Bacteriol. 173, 636–641
22. Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
23. Lineweaver, H., and Burk, D. (1934) J. Am. Chem. Soc. 56, 658–666
24. Nakamura, A., Fukumori, F., Horinouchi, S., Masaki, H., Kudo, T., Uozumi, T., Horikoshi, K., and Beppu, T. (1991) J. Biol. Chem. 266, 1579–1583
25. Nosho, Y., and Sekiguchi, T. (1990) Trends Biochem. Sci. 16, 1–20
26. Garwood, J., Ogush, J., and Kates, R. (1987) J. Biol. Chem. 262, 79–83
27. Gaboriaud, C., Bissery, V., Benchetrit, T., and Mornon, J. P. (1987) FEBS Lett. 224, 149–155
28. Henrisell, B., Rainbody, E., Tran, V., and Mornon, J. P. (1990) Comput. Appl. Biosci. 6, 3–5
29. Numata, K., Muro, M., Akutu, N., Nosho, Y., Yamagishi, A., and Oshima, T. (1995) Protein. Eng. 8, 39–43
30. Molgat, G. F., Donald, L. T., and Duckworth, H. W. (1992) Arch. Biochem. Biophys. 298, 238–246
31. Mas, T. M., Chen, C. Y., Itzkan, R. A., and Riggs, A. D. (1986) Science 233, 788–790
32. Frandsen, T. P., Dupont, C., Lehmbeck, J., Stoffer, B., Siers, M. R., Honzatko, R. B., and Svensson, B. (1994) Biochemistry 33, 13808–13816
33. Siers, M. R., and Svensson, B. (1994) Protein Eng. 7, 1479–1494
34. Olsen, K., Christensen, U., Siers, M. R., and Svensson, B. (1993) Biochemistry 32, 9686–9693
35. Siers, M. R., and Svensson, B. (1993) Biochemistry 32, 1113–1117