RESEARCH COMMUNICATION

Construction and characterization of a chimeric β -glucosidase

Ajay SINGH, Kiyoshi HAYASHI,* Truong T. HOA, Yutaka KASHIWAGI and Ken TOKUYASU

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

The amino acid sequences of β -glucosidases from *Cellvibrio gilvus* and *Agrobacterium tumefaciens* show significant similarity in most of the parts. However, the pH/temperature optima and stabilities of the two enzymes are quite different. *C. gilvus* β -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. To analyse these properties further, a chimeric β -glucosidase was constructed by replacing a

segment from the C-terminal region of *C. gilvus* β -glucosidase gene with that of *A. tumefaciens*. The partially purified chimeric enzyme was characterized with respect to pH/temperature activity and stability and substrate affinity. Our results suggest that C-terminal segment(s) might be important in β -glucosidase specificity, and shuffling of even a small segment of gene in this region might significantly alter or improve the enzymic properties such as thermal stability.

INTRODUCTION

The enzyme β -glucosidase (EC 3.2.1.21) catalyses the hydrolysis of alkyl and aryl β -D-glucosides (methyl β -D-glucoside and *p*-nitrophenyl β -D-glucoside), as well as glycosides containing only carbohydrate residues (cellobiose). On the basis of substrate specificity, β -glucosidases can be classified as aryl- β -glucosidases, cellobiases, and those hydrolysing both aryl β -glucosides and oligosaccharides. The last group is often found in cellulolytic micro-organisms [1,2]. It is one of the components of the cellulase enzyme complex required for the hydrolysis of cellulose to glucose by catalysing the final step, which converts cellobiose into 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 for β -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 similarity. Analysis of structure–function relationships may be facilitated by the formation of chimeric genes/enzymes produced by gene fusion [11]. However, no work has been done on chimeric β -glucosidases so far.

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 and xylanase systems of this organism have been investigated in our laboratory [13,14], as well as the cloning and analysis of the genes coding these enzymes [15,16]. During deletion analysis of the *C. gilvus* β -glucosidase gene (K. Hayashi and T. T. Hoa, unpublished work), we observed that the deletion of a fragment of more than 70 bp from the C-terminal part resulted in the loss of enzyme activity. Thus the C-terminal region seems to be important for β -glucosidase activity. The amino acid sequences of *C. gilvus* β -glucosidase gene show significant homology in most of the parts with those of a β -glucosidase gene from *Agrobacterium tumefaciens* [17]. Despite this homology, their enzymic properties, especially pH–activity and thermal stability, are quite different. To analyse these proper-

ties further, a chimeric β -glucosidase was constructed between them by substituting a small segment from one enzyme in the C-terminal region of the other, and the enzyme characteristics of parental and chimeric enzymes were compared. To the best of our knowledge, this is the first report on chimeric β -glucosidases.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli NM522 *hsd* Δ 5 Δ (*lac-pro*) [*F'* *pro*⁺ *lac* *IqZ* Δ M15] and *E. coli* DH5 α *F'* ϕ 80d *lacZ* Δ M15 *rec* A1 *end* A1 *gyr* A96 *thi*-1 *hsd* R17 (*r*_k[–] *m*_k⁺) *sup* E44 *rel* A1 *deoR* Δ (*lac* *ZYA-arg* *F*)U169 were hosts for β -glucosidase genes from *C. gilvus* and *A. tumefaciens* respectively. pCG5 and pcbg1 were the recombinant plasmids carrying *C. gilvus* [15,16] and *A. tumefaciens* [17] β -glucosidase genes respectively. Recombinant DNA techniques and methods for agarose-gel electrophoresis were followed as described by Sambrook et al. [18]. The clones producing β -glucosidase were detected by fluorescence from 4-methylumbelliferone on a u.v. trans-illuminator after growing on LB-agar plates containing 1 mM 4-methylumbelliferyl β -glucoside [15].

DNA sequencing

Nucleotide sequences of the chimeric gene were determined with a Taq Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems) by using primer ²³⁹⁴CCGATGTCCACCAATGG and sequencing on a DNA Sequencer (model 373A, Applied Biosystems).

Production and purification of β -glucosidases

Cultures were grown aerobically in LB broth containing ampicillin (50 μ g/ml) at 30 °C. The bacterial cells were harvested in the late-exponential phase of growth by centrifugation, and sonicated with a Branson Sonifier model 250/450. Cell debris was separated by centrifugation, and the supernatant was obtained as a crude enzyme preparation.

C. gilvus, *A. tumefaciens* and chimeric β -glucosidase preparations were partially purified by ion-exchange chromatography.

* To whom correspondence should be addressed.

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession number D42076.

C. gilvus and the chimeric enzyme preparation were applied to an f.p.l.c. system (Pharmacia LKB Biotechnology) 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. For *A. tumefaciens* β -glucosidase, 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 Bistris-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.

Hydrophobic cluster analysis was done by the methods of Gaborioud et al. [19] and Henrissat et al. [20].

β -Glucosidase assay

In the standard assay, a sample of suitably diluted enzyme solution was incubated in the reaction mixture containing 25 mM Mops buffer (pH 7.0) and 2 mM *p*-nitrophenyl β -glucoside for 30 min at 30 °C. The reaction was stopped by 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 μ mol of *p*-nitrophenol/min under the above conditions.

Enzymes and chemicals

All the restriction enzymes used in this study were purchased from commercial suppliers (TaKaRa; BioLabs) and used according to the manufacturers' recommendations. Substrates for enzyme assays (*p*-nitrophenyl β -D-glucoside and 4-methylumbelliferyl β -D-glucoside) were obtained from Sigma, and ion-exchange columns from Pharmacia. All other chemicals used were of reagent grade.

RESULTS AND DISCUSSION

Construction of chimeric β -glucosidase

β -Glucosidase genes of *C. gilvus* [15,16] and *A. tumefaciens* [17] have been previously cloned and their nucleotide sequences have

been reported. Despite the significant homology of the deduced amino acid sequences (Figure 1), the enzymic features, especially the heat stability and pH-activity profile of the two β -glucosidases, were quite distinct. To analyse these differences further, as well as to see the effect of shuffling of homologous segments in the C-terminal region, a chimeric β -glucosidase derived from two genes was constructed. The plasmid pCG5 was used as a vector in order to construct the chimera. A schematic representation of the structure of the chimeric β -glucosidase gene is shown in Figure 2. A 647 bp fragment (insert DNA) was obtained by digestion of *pcbg1* with *Ava*I. This fragment was recovered from the agarose gel, purified and blunted. pCG5 was digested with *Sty*I, blunted, and dephosphorylated before ligation with insert DNA. The ligated plasmid was transformed into *E. coli* and the transformants exhibiting enzyme activity on LB-agar plates containing 4-methylumbelliferyl β -glucoside were isolated. The size of the chimeric plasmid was confirmed by digestion with *Eco*RI and *Eco*RV (Figure 3). The nucleotide sequences of the chimeric gene were also confirmed by sequencing the relevant part of the chimeric gene (Figure 4).

Partial purification and characterization

During the course of the purification of parental and chimeric β -glucosidases, marked differences in their behaviour in ion-exchange chromatography were observed. When a Resource S cation-exchange column (1 ml) was used at pH 5.0, *C. gilvus* and chimeric (CH-15) β -glucosidases were eluted at 260 mM and 240 mM NaCl respectively, whereas *A. tumefaciens* enzyme was not adsorbed by the column. On the other hand, Resource Q anion-exchange chromatography was successfully used to elute *A. tumefaciens* β -glucosidase at 160 mM NaCl at pH 6.5. The chimeric β -glucosidase containing most of the regions from *C. gilvus* showed similar behaviour to that of *C. gilvus* β -glucosidase in ion-exchange chromatography. To determine properties, parental and chimeric enzymes were purified by f.p.l.c. on larger scale using either SP-Sepharose (cation-exchange) or Q-Sepharose (anion-exchange) columns. It was possible to achieve about 40-fold purification of crude β -glucosidase preparations from *C. gilvus* and CH-15, and about 60-fold purification of the enzyme from *A. tumefaciens*.

The pH-activity and stability profiles of *C. gilvus* and *A.*

AT	ALRFGVEKPLGDAGIAEAVETARKSDIVLLLVGREGEWDTEGLDLPDMRLPGRQEELIEA	578
CG	IQAQAPNAKVVFDDGRDPAARAARVAAGADVALVFANQWIGEANDAQTALALPDGQEELITS	510
AT	VAETNPVVVVLQGTGGPIEMPWLKGVRAVLQMWYPGQELGNALADVLFQDVEPAGRLPQT	638
CG	VAGANGRTVVVLQGTGGPVTMPWLARVPAVLEAWYPGTSGGEAIAANVLFQAVNPSPGHLPAT	570
AT	FPKALTD-NSAITDDPSIYPGQDGHVRYAEGIFVGYRHHDTREIEPLFPFGFLGYTRFT	697
CG	FPQSEQQQLPRPKLDGDPKNPELQFAVDYHEGAAGVGYKWFDLKGHKPLFPFGHLSYTTFA	630
AT	WGAPQLSGTEMGADGLTVDVTNIGDRAGSDVVQLYVHSPNARVERPFKELRAFAKLKL	757
CG	YSG--LSG-QLKDGRHLVRFKVTNTGNVAGKDVPAVYAAPMSTKWEAP-KRLAAWSKVAL	686
AT	APGATGTAVLKIAPRDLAYFDVEAGRFRADAGKYELIVAASAIDIRA-SVSIHLPVDHVM	816
CG	LPGETKEVEVAEPRVLAMFDEKSRTWRRPKGKIRLTAEADASAANATSVTVLPASTLD	746
AT	EP	818
CG	ARGRAR	752

Figure 1 Amino acid sequences of *A. tumefaciens* (AT) and *C. gilvus* (CG) β -glucosidases in the C-terminal region

Identical and similar amino acid residues are designated by * and . respectively. The chimeric enzyme was constructed by shuffling the region marked by arrowheads.

Figure 2 Schematic representation of parental and chimeric β -glucosidase genes

Light- and dark-shaded bars represent regions derived from *C. gilvus* and *A. tumefaciens*, respectively.

Figure 3 Confirmation of chimeric plasmid by agarose-gel electrophoresis following *EcoRI/EcoRV* digestion

Lane 1, *HindIII* digest of λ DNA marker; lane 2, *EcoRI/EcoRV* digest of pCG5. Chimeric plasmid is shown in lane 2 with 2.9-, 1.3- and 1.2-kb bands. The 1.2-kb band was created by the *EcoRV* site in the inserted gene.

```

2394 GAGCAAGGTC GCCCTGCTGC CGGGCGAGAC CAAGccgggc ggcaccgga cggcggtgct
2454 gaagatcgct cctcgagact tggcttactt cgatgtcgag gccggtcgtt tccgggctga
2514 tgagggaagc tacgagctga tcgtggcggc cagcgccatc gatatccggg caagcgtaag
2574 tattcacttg ccggctgcatc atgtgatgga gccgtag
  
```

Figure 4 Nucleotide sequences of the relevant part of the chimeric gene as determined by Taq dye deoxy terminator cycle sequencing kit (Applied Biosystems)

Lower-case letters indicate the substituted sequences from *A. tumefaciens*.

Figure 5 pH-activity (a), pH-stability (b), temperature optima (c) and heat-stability (d) profiles of parental and chimeric β -glucosidases

The pH was adjusted with buffers: citrate (pH 3.0–4.0); Mes (pH 5.0–6.8); Mops (pH 7.0–8.0); 2-(*N*-cyclohexylamino)ethanesulphonic acid (pH 9.0–10.0). For pH-stability experiments, enzyme was incubated at different pH values for 1 h at 25 °C. For heat-stability experiments, each enzyme at its optimum pH was treated at different temperatures for 1 h. The residual activities were measured under standard assay condition. ●, *C. gilvus*; ■, CH-15; ▲, *A. tumefaciens*.

tumefaciens β -glucosidases are shown in Figure 5. *C. gilvus* enzyme exhibited the maximum activity at pH 6.2–6.4, about 66% at pH 7.0 and no activity at pH 9.0. On the other hand, the optimum pH of the *A. tumefaciens* enzyme was 7.2–7.4, with about 86% activity at pH 5.0 or 8.0. The pH-activity profile of the CH-15 enzyme was similar to that of *C. gilvus*, with pH optimum at 6.0. Both *C. gilvus* and CH-15 enzymes were stable between pH 4 and 9, whereas *A. tumefaciens* enzyme was stable between pH 5 and 10. Thus no marked differences in pH-activity/stability characteristics between *C. gilvus* and CH-15 enzymes were evident.

C. gilvus and *A. tumefaciens* β -glucosidases showed marked differences in their temperature optima also (Figure 5). *C. gilvus* enzyme was optimally active at 35 °C, but had little activity at 50 °C. *A. tumefaciens* β -glucosidase exhibited maximum activity at 60 °C, and 70% of its maximum activity was still evident at 65 °C. The chimeric β -glucosidase exhibited a significant difference in temperature optimum from the other two enzymes. The chimeric enzyme was optimally active at 40–45 °C, showing an intermediate temperature optimum between the *C. gilvus* and *A. tumefaciens* enzymes. Heat-stability experiments revealed that *C. gilvus* enzyme was completely active up to 30 °C, retained about 80% of its maximum activity at 35 °C, and was completely inactivated at 55 °C. *A. tumefaciens* enzyme was stable up to 55 °C, and even at 65 °C, 60% of its maximum activity was retained. Chimeric β -glucosidase was stable up to 35 °C, and retained 60% of its maximum activity at 45 °C.

It is noteworthy that the heat-stability was changed distinctly even by replacing only about a 12% segment of *C. gilvus* β -glucosidase gene with that of *A. tumefaciens*. The temperature at which 50% loss of activity from *C. gilvus*, *A. tumefaciens* and CH-15 enzymes occurred was 41 °C, 67 °C and 47 °C respectively.

Figure 6 Hydrophobic cluster analysis of *C. gilvus* and chimeric β -glucosidase

Analysis of only relevant parts of the native and chimeric enzyme is presented. Letters circled and underlined are hydrophobic amino acids, whereas letters in squares are proline and glycine separating hydrophobic clusters.

Thus heat-stability may be influenced by only a few amino acid substitutions. Hydrophobic interactions inside the protein molecule are one of the most important factors in stabilizing protein structure. Hydrophobic cluster analysis 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 enzyme (Figure 6). These substitutions might be important for heat-stability.

The chimeric enzyme retained an efficient active site, as shown by the fact that its affinity for *p*-nitrophenyl β -glucoside was similar to that for the native enzymes from which it was derived. The K_m value of the CH-15 enzyme (2.2 ± 0.26 mM) was indeed lower than the corresponding value for *C. gilvus* (3.5 ± 0.35 mM) and *A. tumefaciens* (4.5 ± 0.23 mM) enzymes, indicating a greater substrate affinity. The K_m values of the chimeric citrate synthases have similarly been found to be lower than those of the parental enzymes [21]. However, substrate affinity was found to decrease by about 2-fold in active human–yeast chimeric phosphoglycerate kinase engineered by domain interchange [22].

It has been reported that the deletion of about 100 amino acid residues near the C-terminal region of the α -amylase gene did not affect enzyme activity [23]. Similarly, a large part of the C-terminal (about 46 kDa) of cellulase from alkalophilic *Bacillus* sp. No.1139 was found not to be necessary for enzyme activity [24]. In contrast, cyclomaltodextrin glucanotransferases lacking 30 amino acids [25] and an endoglucanase lacking 75 amino acids [26] from the C-terminal end showed no enzyme activity [25]. Our results indicate that the C-terminal region is important for β -glucosidase activity and the changes in enzymic properties on substitution of the C-terminal segment might be related to enzyme specificity. Therefore the C-terminal region of this enzyme also plays an important role in determining its characteristics. Chimeric enzymes with altered or improved enzymic

properties can be prepared in a convenient and effective way by manipulating this region.

An STA Fellowship provided by JISTEC/JRDC, Japan, to A. S. is gratefully acknowledged. We are grateful to Dr. L. A. Castle and Dr. R. O. Morris, University of Missouri, for useful discussions and for providing the recombinant plasmid carrying the β -glucosidase gene from *A. tumefaciens*. Ms. Chika Aoyagi provided excellent technical assistance. Thanks are also due to Dr. J. Snape for critically reading the manuscript.

REFERENCES

- 1 Beguin, P. (1990) Annu. Rev. Microbiol. **44**, 219–248
- 2 Paavilainen, S., Hellman, J. and Korpela, T. (1993) Appl. Environ. Microbiol. **59**, 927–932
- 3 Ryu, D. D. Y. and Mandels, M. (1980) Enzyme Microb. Technol. **2**, 91–101
- 4 Shewale, J. G. (1982) Int. J. Biochem. **14**, 435–443
- 5 Glick, B. R. and Pasternak, J. J. (1989) Biotechnol. Adv. **7**, 361–386
- 6 Kuhad, R. C. and Singh, A. (1993) Crit. Rev. Biotechnol. **13**, 151–172
- 7 Love, D. R. and Streiff, M. B. (1987) Bio/Technology **5**, 384–387
- 8 Beguin, P., Gilkes, N. R., Kilburn, 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., Kilburn, D. G., Miller, R. C. 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. (1991) Methods Enzymol. **202**, 687–706
- 12 Storwick, W. O. and King, K. W. (1960) J. Biol. Chem. **235**, 303–307
- 13 Haga, K., Kitaoka, M., Kashiwagi, Y., 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., Ohmia, K., Kobayashi, Y., Hoshino, S., Sakka K. and Karita, S. eds.), pp. 368–377, UNI Publishers Co., Tokyo
- 17 Castle, L. A., Smith, K. D. and Morris, R. O. (1992) J. Bacteriol. **174**, 1478–1486

-
- 18 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York
 - 19 Gaborioud, C., Bissery, V., Benchetrit, T. and Mornon, J. P. (1987) *FEBS Lett.* **224**, 149–155
 - 20 Henrissat, B., Raimboud, E., Tran, V. and Mornon J. P. (1990) *Comput. Appl. Biosci.* **6**, 3–5
 - 21 Molgat, G. F., Donald, L. J. and Duckworth, H. W. (1992) *Arch. Biochem. Biophys.* **298**, 238–246
 - 22 Mas, M. T., Chen, C. Y., Hitzman, R. A. and Riggs, A. D. (1986) *Science* **233**, 788–790
 - 23 Yamane, K., Hirata, Y., Furusato, T., Yamazaki, H. and Nakayama, A. (1984) *J. Biochem. (Tokyo)* **96**, 1849–1858
 - 24 Fukumori, F., Kudo, T. and Horikoshi, K. (1987) *FEMS Microbiol. Lett.* **40**, 311–314
 - 25 Kaneko, T., Song, K.-B., Hamamoto, T., Kudo, T. and Horikoshi, K. (1989) *J. Gen. Microbiol.* **135**, 3447–3457
 - 26 Ohmiya, K., Deguchi, H. and Shimizu, S. (1991) *J. Bacteriol.* **173**, 636–641
-

Received 1 November 1994; accepted 11 November 1994