Chimeric ß-Glucosidase between *Cellvibrio gilvus* and *Ruminococcus albus* Enzymes

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ABSTRACT

Based on homology search in the amino acid sequences, a chimeric ß-glucosidase with enzyme activity has been prepared by replacing C-terminal region of Thr509-Arg646 from Cellvibrio gilvus *with N-terminal region of Arg160-Gly283 from* Ruminococcus albus*. The chimeric enzyme showed broader optimum pH and slightly higher heat stability compared with original* C. gilvus *enzyme.*

Key words: Chimeric, *Cellvibrio gilvus, Ruminococcus albus*, ß-glucosidase

INTRODUCTION

Cellobiose rather than glucose has been accumulated when *Cellvibrio gilvus* ATCC 13127 was cultivated in a medium containing acid swollen cellulose. It was found that this unique accumulation of the disaccharide has been caused by a specificity of ß-glucosidase (EC 3.2.1.21) of this strain, belonging to family 3 of the glycosyl hydrolase; substantially lower activity toward cellobiose than cellotriose, cellotetraose, cellopentaose and cellohexaose (Kashiwagi *et al.*, 1991), and no transglycosidation activity (Watt *et al*., 1998). Accumulated cellobiose has been considered to be utilized in the cell by hydrolysing it to glucose and glucose 1-phosphate by cellobiose phosphorylase (EC 2.4.1.20) (Liu *et al.*, 1998). As an initial step to understand this unique substrate specificity of the ß-glucosidase, chimeric enzyme has been prepared and characterized.

MATERIALS AND METHODS

Chemicals and enzymes: 4-Methylumbelliferyl ß-glucopyranoside (4-MUG) was supplied by Sigma (St. Louis, Mo, USA). Restriction enzymes of *Pmac*I, *Bcl*I, *Bsa*JI, *Hin*P1I and *Eco*RI were obtained from New England Biolabs (Beverly, MA, USA). Kits for deletion, blunting and ligation were obtained from Takara (Tokyo, Japan). DNA fragments extracted from agarose were purified by using Sephaglas Band Prep supplied by Pharmacia (Uppsala, Sweden).

Bacterial strains and plasmids: *E. coli* NM522 was hosts for ß-glucosidase gene from *C. gilvus. E. coli* C600 has been used for the gene from *R. albus* (Honda *et al*., 1988, Ohmiya *et al.*, 1990). *E. coli* JM109 was used for transformation. Plasmid *pCG5* and *pMU1* were the recombinant plasmids carrying *Cellvibrio gilvus* (Kashiwagi *et al*., 1993) and *Ruminococcus albus* ß-glucosidase genes, respectively.

DNA manipulations: Plasmid DNA was prepared by using a kit (QIAprep Spin Miniprep, Qiagen, USA). The digestion by restriction enzymes was carried out in appropriate buffer at the concentration of 1-10 units per µg DNA for 1-3 h at appropriate temperature. The completion of the reaction was confirmed by agarose gel

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electrophoresis. After obtaining the insertion DNA fragment from *pMU1* and deleted plasmid from *pCG5*, they were blunted, ligated and then transformed *to E. coli* JM101 cells. The transformants were grown on LB-medium containing 50 µg/ml of ampicillin and 1 mM of 4-MUG. Selection of transformants harboring plasmid of *pCG5H* was carried out by detecting the enzyme activity under UV illuminator.

ß-Glucosidase assay: Cells harboring plasmid *pCG5H* were cultivated at 30 °C in LB medium for 20 h. Cell free extract obtained by sonication was subjected to the assay by using 4-MUG as substrate (Kashiwagi *et al*., 1991). Hundred times more accurate analysis than the previously reported measurement (Kashiwagi *et al*., 1991) has been achieved by kinetic analysis with monitoring the increase of the absorbance at 357 nm at 1 min interval for 20 min by using a spectrophotometer (DU-7400, Beckman, USA).

RESULTS AND DISCUSSION

Construction of chimeric gene: Homology search in amino acid sequences of ß-glucosidase from *C. gilvus* has been carried out by using data base of SWISS PROT. Several kinds of enzyme produced by yeast, fungi and bacteria have been selected to be significantly homologous. In order to produce chimeric enzyme, ß-glucosidase from *R. albus* has been selected among enzymes based on the following reasons: A bacterium is preferred because of less chance of post-translational modification such as addition of saccharides to the protein. N-terminal region and C-terminal region of ß-glucosidase from *C. gilvus* showed homology to C-terminal and N-terminal region from *R. albus*, respectively, as shown in Fig 1.

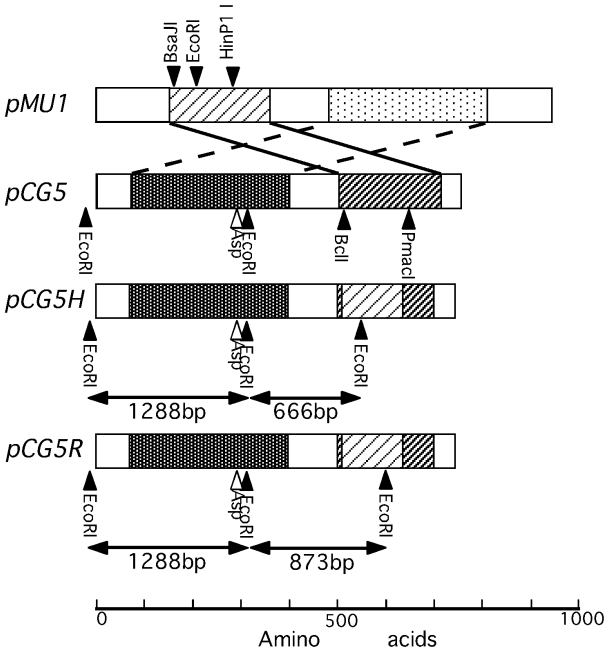

Figure 1: Homology in amino acid sequence of ß-glucosidase between *C. gilvus* and *R. albus*.

Two domains showing homology, shaded region and hatched region, were observed in inverted position. Estimated catalytic site of Asp291 is shown as Asp. ß-glucosidase from *R. albus*, *C. gilvus*, chimeric enzyme and reverse chimeric enzyme are *pMU1*, *pCG5*, *pCG5H* and *pCG5R*, respectively. This inversion of homologous region was only observed in the two enzymes produced by *Butyrivibrio fibrisolvens* (Lin *et al.*, 1990) and *R. albus*. Since expected catalytic center of Asp291 is located in the N-terminal region of *C. gilvus* enzyme, C-terminal region has been selected for preparation of a chimeric enzyme. Considering the translation frame and homologous region of both genes, the region between *Bcl*I and *Pmac*I sites (BP region) coding Thr509-Arg646, 138 amino acid residues out of 752, in the plasmid of pCG5 and the region between *Bsa*JI and *Hin*P1I sites (BH region) coding Arg160 Gly283, 124 amino acid residues out of 947, in the plasmid of pMU1 have been selected to create a chimeric enzyme gene (*pCG5H*). The replaced region showed amino acid identitiy of 34%. The construction of chimeric gene of ß-glucosidase has been carried out as described in the material and method. Since there is one *Eco*RI site in the inserted fragment of BH region as shown in Fig 1,

pCG5H and *pCG5R* were confirmed by *Eco*RI digestion. The DNA fragments of 666 bp in case of *pCG5H* and 873 bp in case of *pCG5R* have been observed as shown in Fig. 2.

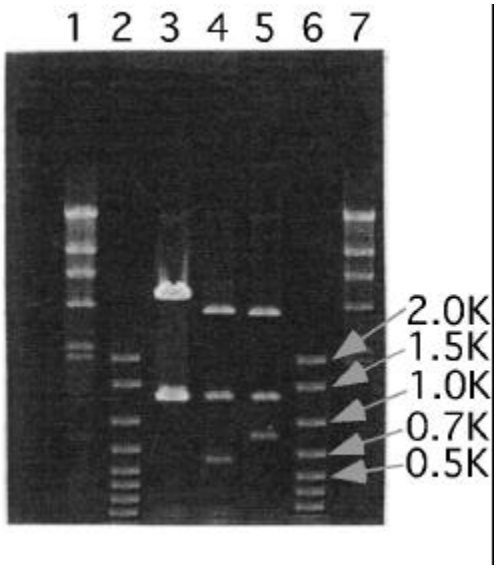

Figure 2: Confirmation of chimeric plasmid by agarose gel electrophoresis.

Lane1 and 7, *Hind*III digest of λphageDNA maker; Lane 2 and 6, DNA maker of 2.0, 1.5, 1.0, 0.7, 0.5, 0.4, 0.3, 0.2, 0.1 Kbp; Lane 3, *pCG5*; Lane 4, *pCG5H* (chimeric enzyme); Lane 5, *pCG5R* (reversed insert). After digestion of plasmid with *Eco*RI, DNA fragments of 663 bp in Lane 4 (*pCG5H*) and 873 bp in lane 5 (*pCG5R*) were observed.

Characterization of chimeric gene: It was found that C-terminal regions is essential to keep the enzyme activity based on the following observation. The truncated mutant at PB region in the plasmid of *pCG5* showed no enzyme activity. The plasmid having the BH fragment in the inverted position (*pCG5R*) showed no enzyme activity. Deletion mutant of 50-300 nucleotide bases in the C-terminal region of *pCG5* resulted in complete loss of the enzyme activity. Especially, it is reported that the five amino acid residues containing 3 arginine residues at the C terminal end, RGRAR are quite important for the enzyme folding and activity (Kim et al., 1998). However, replacement of PB region with homologous peptide of BH region was successful in obtaining the enzyme with activity. The chimeric enzyme folded in the transformat cell without special help such as co-expression with GroEL/ES (Machida et al., 1998). After extracting enzyme by sonic disruption of cells, the activity of chimeric (pCG5H) and original (pCG5) enzyme has been measured by changing pH and temperature.

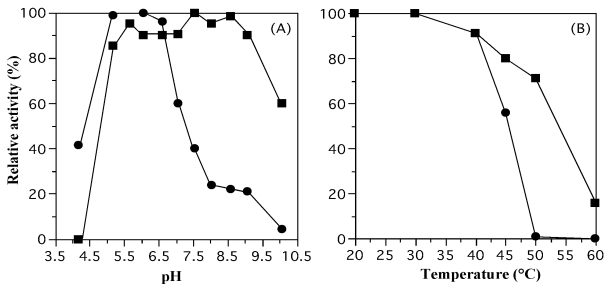

Figure 3: Optimum pH (A) and thermal stability (B) of chimeric enzyme from *pCG5H* (■) and original enzyme from *pCG5* (●).

The optimum pH of the chimeric enzyme was found to be 5-9 which is broader than original enzyme as shown in Fig. 3(A). Temperature stability of chimeric enzyme has increased by 5 °C as shown in Fig. 3(B). The optimum pH and temperature of the enzyme from *R. albus* were reported to be 6.0 and 35 °C, respectively (Honda *et al.*, 1988). Most chimeric enzymes prepared so far have high homology in amino acid sequences in the replaced region; 83% (Kaneko *et al.*, 1989), 70% (Nakamura *et al.*, 1991). It is quite interesting to know that the enzyme is still active after replacement of the region with relatively lower homology of 34%. It is also interesting to know that the heat stability has slightly improved by making chimeric enzyme.

These result suggest that C-terminal region of *C. gilvus* ß-glucosidase and N-terminal region of the *R. albus* ß-glucosidase may fold in a similar shape since the replacement of this region resulted in the enzyme with activity. It also shows that enzymes with improved characteristics can be designed through preparing chimeric enzyme.

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RESUMO

Baseado na busca de homólogos na seqüência de aminoácidos, uma ß glucosidase - quimérica com atividade enzimática foi preparada para substituir C-terminal na região de Thr509-Arg646 de *Cellvibrio gilvus* com N-terminal na região de Arg160-Gly283 de *Ruminococcus albus*. A enzima quimérica mostrou pH ótimo mais amplo e estabilidade levemente mais alta ao calor comparada com a enzima original de *C. gilvus.*

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