Short Communication

EXPRESSION OF THREE β -GLUCOSIDASE GENES FROM *ARTHROBACTER* SP. GJM-1 IN *ESCHERICHIA COLI*

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β -Glucosidases are a heterogeneous group of enzymes with respect to their substrate specificity or their protein structure (13). Most β -glucosidases catalyze the hydrolysis of β -1,4-glycosidic linkages between glucose and alkyl-, aryl- (e.g., methylumbelliferyl- β -D-glucoside = MUG, *p*-nitrophenol- β -D-glucopyranoside = PNPg) or saccharide groups (cellobiose) (10). However Ait et al. (1) described a β -glucosidase from the thermophilic strain *Clostridium thermocellum* which hydrolyzes all four possible β -glycosidic bonds of glucose dimers, like sophorose (β -1,2), laminarribose (β -1,3), cellobiose (β -1,4) and gentibiose (β -1,6).

β -Glucosidases are essential components of the multienzyme systems of cellulolytic microorganisms which utilize crystalline and/or soluble cellulosic materials. During the degradation of cellulose by endo- and exoglucanases cellobiose is released and accumulated. This, in turn, inhibits cellulolytic activities of both glucanases. Therefore, the conversion of cellobiose to glucose by β -glucosidases results in a more efficient cellulolysis (3). Because of their biological importance, β -glucosidases have been isolated and characterized from both eukaryotic and prokaryotic organisms (4, 7).

Here, we describe the cloning, expression and first characterization of three β -glucosidase genes from the Gram-positive strain *Arthrobacter* sp. GJM-1 in *Escherichia coli*. Members of the genus *Arthrobacter* represent a major group of Gram-positive soil bacteria. Many *Arthrobacter* species show a versatile metabolism, e.g., some are able to degrade herbicides, pesticides, chitin, and different aromatic compounds and some produce antibiotics, phytohormones, riboflavin and polysaccharides. Vrsanska et al. (15) has characterized two β -glucanases from

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Arthrobacter species. One enzyme is able to hydrolyze β -1,3-, the other β -1,6-glycosidic bonds.

In the latest description of the genus *Arthrobacter*, it is stated emphatically that cellulose is not decomposed (6). However, Menge et al. (9) have isolated cellulases from *Arthrobacter* sp. GJM-1 and used them to produce protoplasts from *Digitalis lanata*. They detected three different enzyme activities during the fermentation of crystalline cellulosic substrates: exo- β -1,4-glucanase, endo- β -1,4-glucanase and β -glucosidase. Until now, no molecular genetic studies of the cellulolytic system of this bacterial strain have been reported.

Arthrobacter sp. GJM-1 was grown at 30°C for 48 h in medium A (150 mM $(\text{NH}_4)_2\text{SO}_4$, 7 mM MgSO_4 , 120 mM CaCl_2 , 200 mM Na_2HPO_4 , 10 mM KH_2PO_4 , 0.015 mM FeSO_4 , 0.75 g/l yeast extract, 3 g/l crystalline cellulose). *E. coli* ER1451 (11) was cultivated in Luria broth (LB-medium, 12) at 37°C for 12 h. After 48 h, 2 ml of the *Arthrobacter* sp. GJM-1-“starter” culture was transferred to 200 ml of fresh medium A and incubated at 30°C for two days. Bacterial cells were pelleted by centrifugation. Cells were suspended in 1/4 volume of *n*-butanol, sedimented and resuspended in 30 ml of buffer 1 (50 mM Tris-HCl, 20 mM EDTA, pH 8). Cells were collected once again by centrifugation and resuspended in 30 ml of buffer 1. Lysozyme was added to a final concentration of 1 mg/ml. After incubation for 1–2 h at room temperature, sodium dodecyl sulfate (SDS) was added to a final concentration of 2%. After a further incubation for 15 min at 65°C, NaCl was added to a final concentration of 1 M and the mixture was incubated for 30 min at 37°C. DNA was purified by chloroform extraction and precipitation with ethanol at –20°C. Precipitated DNA was spooled onto a glass rod and then suspended in 3 ml of buffer 2 (10 mM Tris-HCl, 10 mM EDTA, pH 7.5). After treatment with RNase A (final concentration: 50 $\mu\text{g/ml}$) for 2 h at 37°C and phenol/chloroform purification, DNA was ethanol-precipitated and finally redissolved in an appropriate volume of TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

Arthrobacter DNA was partially digested with *Sau*3A and separated by agarose gel electrophoresis. DNA fragments between 5- and 10-kb in size were eluted from the gel and ligated to *Bam*HI-cleaved vector pUC18. *E. coli* ER1451 was transformed by the ligation mixture and transformants were selected on LB-plates containing ampicillin (Ap; 100 $\mu\text{g/ml}$) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Xgal; 40 $\mu\text{g/ml}$).

Three thousand recombinant clones were screened for β -glucosidase activity using the chromogenic substrates *p*-nitrophenol- β -D-glucopyranoside and *p*-nitrophenol- β -D-cellobiose (PNPC) according to the following protocol: 3 ml of 12 h cultures of recombinant clones were grown in LB-medium with ampicillin at 37°C. After the addition of isopropyl- β -D-thiogalactoside (IPTG) to a final concentration of 0.2 mM and further incubation for 2–4 h, bacteria from 1 ml of the culture were sedimented. The pellet was suspended in 30 μl of 0.1% Triton X-100 and mixed with 10 μl of the appropriate substrate (8 mM PNPG or PNPC suspended in 100 mM sodium citrate buffer, pH 5.2). β -Glucosidase activity of the clones was

detected by the release of *p*-nitrophenol after 4–6 h at room temperature. Three *E. coli* clones containing recombinant plasmids (named pCK2, pCK3 and pCK4) hydrolyzed the substrate PNPG. In addition, plasmid pCK3 carries DNA coding for a PNPC-cleaving activity. Inserts of the plasmids pCK2, pCK3 and pCK4 are 5.3-kb, 10.5-kb and 8.0-kb in size, respectively. Physical maps were constructed for the three recombinant plasmids and are shown in Fig. 1. Restriction analyses, including the comparison of single and double digests, were generated using the following enzymes: *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Hinc*II, *Hind*III, *Kpn*I, *Pst*I, *Sac*I, *Sma*I, *Sph*I and *Stu*I. The results of these analyses, especially the comparison of the *Eco*RI- and *Hind*III-digests, showed no significant homology with the three *Arthrobacter* inserts. To confirm this result, a Southern hybridization experiment was carried out. DNA of the plasmids pCK2 and pCK4 was digested with *Bam*HI and *Kpn*I/*Xba*I, respectively, to separate the vector and the appropriate insert on a 1% agarose gel. The DNA was then blotted to a nylon membrane (HybondTM-N⁺, Amersham International plc., Buckinghamshire, England) in 20×SSC (3 M NaCl, 0.3 M sodium citrate, pH 7) (14). Fluorescein-11-dUTP-labelled uncleaved probes of the three plasmids pCK2, pCK3 and pCK4 were hybridized to this nylon

Fig. 1. Physical maps of the plasmids pCK2, pCK3 and pCK4.

The cloned DNA fragments are presented by solid bars and the plasmid DNA by open bars. Arrows indicate the orientation of the *lac* promoter. Restriction sites are derived from the insert sequences and from the polylinker region of the vector. Enzymes: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; EV, *Eco*RV; Hc, *Hinc*II; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; St, *Stu*I; X, *Xba*I. β -Glucosidase activity was determined by the release of *p*-nitrophenol using the chromogenic substrates PNPG and PNPC. Overnight cultures of the recombinant clones were incubated with IPTG for a few hours, then sedimented and the pellets were resuspended in the appropriate substrate-buffer mixtures (For details see text.).

Fig. 2. Cross-hybridization experiment of the plasmids pCK2, pCK3 and pCK4.

(A) DNA of the plasmids pCK2 (lane 2) and pCK4 (lane 3) was digested with *Bam*HI and *Kpn*I/*Xba*I, respectively, to separate vector and insert sequences on a 1% agarose gel and to blot it to a nylon membrane. The size of the restriction fragments is indicated using *Hind*III-digested λ DNA as size marker (lane 1). (B) Fluorescein-11-dUTP-labelled uncleaved probes of pCK3 (lanes 1 and 2), pCK2 (lanes 3 and 4) and pCK4 (lanes 5 and 6) were hybridized to pCK2 (lanes 1, 3 and 5) and pCK4 (lanes 2, 4 and 6). The arrow denotes the linearized pUC18. There is no cross-hybridization of the three *Arthrobacter* inserts.

membrane overnight at 65°C and washed once in 0.5×SSC/0.1% SDS and three times in 0.2×SSC/0.1% SDS for 15 min at 65°C. Blocking, antibody incubation, washes and signal detection were done according to the protocol of the ECL™ random prime labelling and detection system (Amersham). No cross-hybridization of the three *Arthrobacter* inserts could be detected (Fig. 2). The results of this hybridization experiment and the restriction analysis as well as the diverse substrate specificities of the three recombinant *E. coli* clones suggest the presence of at least three different β -glucosidase activities in *Arthrobacter* sp. GJM-1.

Arthrobacter sp. GJM-1 is able to degrade *p*-nitrophenol- β -D-xylopyranoside (data not shown). However the screening of the three recombinant *E. coli* clones with this substrate shows no xylosidase activity (data not shown). From these results, we propose that *Arthrobacter* sp. GJM-1 contains either another β -glucosidase with an additional xylosidase activity or a distinct xylosidase.

Similar cases of two or more β -glucosidases with different substrate specificities or a low degree of sequence homology within one organism have been documented. Gräbnitz et al. described two β -glucosidase genes from *C. thermocellum*, called *bglA* and *bglB* with similarities to the bacterial β -glucosidase family A (5) and to the fungal β -glucosidases (4), respectively. Like some other bacterial β -glucosidases, the latter exhibits additional β -xylosidase activity.

β -Glucosidase activities from the recombinant *E. coli* clones [pCK2], [pCK3] and [pCK4] were detected within the cells after the separation of the cytoplasmic and periplasmic fractions according to Sambrook et al. (12). These results agree

with data from Menge et al. (9) showing that, in *Arthrobacter*, 90% of the β -glucosidases are located intracellularly. Nearly all bacterial β -glucosidases are associated with the cytoplasm or inner side of the cytoplasmic membrane (2, 8, 10, 16). One exception to this is a β -glucosidase described by Ait et al. (1) from *C. thermocellum*. This enzyme was found in the periplasmic space.

This is the first report on cloning and expression in *E. coli* of three different β -glucosidase activities from an *Arthrobacter* strain. Further experiments are under way to analyze the DNA sequences of the three inserts to look for homologies with the β -glucosidases of other organisms and to investigate the biochemical and biophysical properties of the proteins.

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