

Separation of Levan-formation and Sucrose-hydrolysis Catalyzed by Levansucrase of *Zymomonas mobilis* Using *in vitro* Mutagenesis

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ABSTRACT

A levansucrase (*SacB*) of *Zymomonas mobilis* capable of sucrose hydrolysis but not levan formation was isolated through *in vitro* mutagenesis of cloned *sacB* gene. When the *sacB* mutant gene was expressed in *Escherichia coli* strains, only 50% of the sucrose-hydrolysing activity (2.0 U/mg) was produced, compared to the wild type levansucrase (4.0 U/mg). Sequencing of the *sacB* mutant gene revealed changes of two amino acid residues (Phe-102 to Leu and Trp-261 to Lys in the levansucrase). The absence of mutation at the site of Cys of *SacB* is contradictory to the inhibition kinetics that demonstrated the involvement of Cys in conferring the levan-forming activity to the *SacB*. The present finding is useful in understanding the mechanism of selective modulation of levan-forming (polymerase) activity of levansucrase.

Key words: Levansucrase, polymerase, sucrose, *in vitro* mutagenesis, levan, selective modulation.

INTRODUCTION

Zymomonas mobilis is an ethanologenic bacterium, which produces an extracellular levansucrase (*SacB*), an extracellular sucrose (*SacC*) and an intracellular sucrose (*SacA*) (O'Mullan *et al.*, 1991). Among these, levansucrase (*SacB*) (sucrose: 2,6- β -D-fructan-6- β -D-fructosyl transferase; EC 2.4.1.10) forms levan as a byproduct during growth of *Z. mobilis* in sucrose medium, which reduces the efficacy of ethanol production (Viikari, 1984). Since an improvement in the ethanol yield depended on the loss or reduction of levan-formation in the fermentation medium, an understanding of the levansucrase catalysis is necessary (Kannan *et al.*, 1993). The critical amino acids involved in the separate modulation of the levan-forming ability of *SacB* have been investigated by genetic or biochemical approaches. The *sacB* gene (Gunasekaran *et al.*, 1995), which allowed the easy purification of the enzyme and construction of its variants. The levansucrase expressed in *E. coli* has been purified and characterized encoding levansucrase has been cloned, sequenced and expressed in *Escherichia*

coli (Sangiliyandi & Gunasekaran, 1998). The selective modulation of the levan-forming activity of the levansucrase by Cys has been demonstrated (Sangiliyandi *et al.*, 1999). Among other sources, levansucrase from *Bacillus subtilis* has been well studied. So far, the mutation in *sacB* gene encoding levansucrase resulting in loss of levan-formation without affecting sucrose hydrolysis has been identified only from *B. subtilis* (Chambert & Petit-Glatron, 1991). We report here for the first time, the sequencing of mutant *sacB* gene of *Z. mobilis* encoding levansucrase catalyzing sucrose hydrolysis but not levan-formation and the present results will be helpful in revealing the mechanism of action of levansucrase.

MATERIALS AND METHODS

Bacterial strains and Plasmids: *Escherichia coli* strains BL21 (DE3) (*F*^{hdsS}, *gal*), JM109 (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE*, *relA1*, (*lac*^{proAB}) [*F'* *traD36*, *proAB*, *lacI*^f, *DM15*] and XL1 Blue (*recA1*, *lac*⁻, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *recA1* [*F'* *proAB*,

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lacI^q, *lacDM15*, *Tn10*]); plasmids-pBluescriptKS⁺ (Stratagene -USA) and pLS3(Sangiliyandi & Gunasekaran, 1998) were used.

In vitro mutagenesis: *In vitro* mutagenesis of plasmid DNA was carried out according to Busby *et al.*, (1982) by treating plasmid DNA with 1M hydroxylamine in 50 mM Sodium pyrophosphate (pH 7.0), 100 mM Sodium chloride and 2 mM EDTA at 75°C (2 h). After transforming *E. coli* JM109 with mutagenised pLS3, levansucrase variants were isolated according to Kannan *et al.*, (1993). Preparation of plasmid DNA and sequencing were carried out as described earlier (Gunasekaran *et al.*, 1995).

Expression of levansucrase: *E. coli* strain JM109 or XL1Blue or BL21 was transformed with pLS3 or pLS3105 and grown in M63 medium supplemented with glycerol (20g/l) (MY) or glucose (20g/l) (MG) or fructose (20g/l) (MF) and LB medium, supplemented with ampicillin (50 µg/ml). After 18 h, the cell lysate was assayed for sucrose or levan-forming activity. *E. coli* cultures were also grown in MY medium to OD₆₀₀ of 1.0, induced with the addition of 0.4 mM IPTG and after 4h, the cell lysate was assayed for enzyme activity (Studier & Moffat, 1986).

Enzyme assay: A reaction mixture containing enzyme extract (100 µl), 1M sucrose (200 µl) and sodium acetate buffer (pH 5.0, 700 µl) was incubated at 60°C for 30 min. and the reducing sugar released was estimated as previously described (Gunasekaran *et al.*, 1995). The sucrose activity was expressed as the amount of enzyme required to release one µmol of reducing sugar in one minute. Levan formed was estimated by measuring the fructose residues after acid hydrolysis of levan (Kannan *et al.*, 1993). Protein was measured by the method of Lowry using bovine serum albumin as a standard.

Zymogram analysis: The cellular proteins of *E. coli* BL21 (pLS3 or pLS3105) were separated on native PAGE, the gel was incubated at 30°C for 12 h in 5% (w/v) sucrose. Appearance of a slimy

white band indicates levan-forming activity. Sucrase activity was identified according to O'Mullan *et al.*, (1991).

RESULTS AND DISCUSSION

Screening of levansucrase mutant: The plasmid pLS3 containing *sacB* gene was treated with hydroxylamine and transferred in to *E. coli* JM109. The cell-lysate of the transformants was assayed for sucrose and levan-forming activities. Among 2000 transformants, one produced sucrose-hydrolysing activity but not levan-forming activity. The plasmid containing *sacB* mutant (encoding defective levansucrase) was isolated from this transformant and designated as pLS3105. In the zymogram analysis, the cellular extracts of *E. coli* (pLS3) or *E. coli* (pLS3105) showed the sucrose activity band corresponding to SacB, while a slimy white band of levan corresponding to SacB was showed by cellular extracts of *E. coli* (pLS3) only (data not shown). These results suggested the selective elimination of levan-forming activity of SacB by *in vitro* mutation. Similar type of mutants from *B. subtilis* have been reported but by *in vivo* mutation of the culture using ethylmethane-sulphonate (Lepasant *et al.*, 1974).

Expression and induction of levansucrase: The expression of sucrose-hydrolysing and levan-forming activity of levansucrase in *E. coli* BL21 (pLS3) or *E. coli* BL21 (pLS3105) is shown in Fig. 1. It is obvious that the levan was not formed by the defective *sacB* expressed in *E. coli* BL21 (pLS3105), while a maximum of 2.0 U/mg of sucrose-hydrolysing activity was expressed at 21 h of growth. However, *E. coli* BL21 (pLS3), harboring the wild type *sacB* gene, expressed a maximum of 4.5 U/mg of sucrose activity and 1.8 U/mg of levan-forming activity.

The comparison of expression of *sacB* in *E. coli* (pLS3) and *E. coli* (pLS3105) was carried out by determining the sucrose activity, since the defective levansucrase did not exhibit the levan-forming activity. When pLS3105 or pLS3 was expressed in various *E. coli* strains, grown in LB

or MY medium, a low level of sucrase activity (0.26-1.23 U/mg) was obtained in *E. coli* strain JM109 or XL1Blue compared to *E. coli* BL21 (0.95-4.0 U/mg) (Table 1).

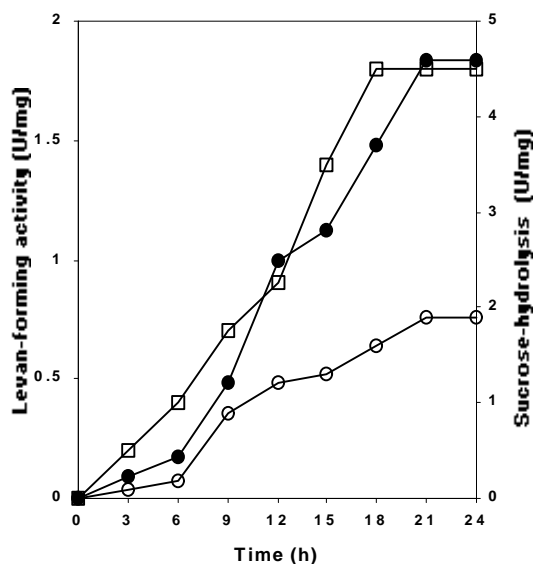


Figure 1. Expression of levansucrase by *E. coli* strains. *E. coli* BL21 (pLS3) and *E. coli* BL21 (pLS3105) were grown in MY medium at 37°C. The cellular extract was assayed for the sucrose-hydrolysing and levam-forming activities. The cellular extract of *E. coli* (pLS3105) did not form levam. Sucrose hydrolysis: (●) *E. coli* (pLS3), (○) *E. coli* (pLS3105). Levam-formation: (□) *E. coli* (pLS3)

The comparison of expression of *sacB* in *E. coli* (pLS3) and *E. coli* (pLS3105) was carried out by determining the sucrase activity, since the defective levansucrase did not exhibit the levam-forming activity. When pLS3105 or pLS3 was expressed in various *E. coli* strains, grown in LB or MY medium, a low level of sucrase activity (0.26-1.23 U/mg) was obtained in *E. coli* strain JM109 or XL1Blue compared to *E. coli* BL21 (0.95-4.0 U/mg) (Table 1). However, *E. coli* (pLS3105) expressed only 50% of sucrase compared to *E. coli* (pLS3), irrespective of growth medium or host strain. Levansucrase synthesis was not inducible with IPTG in *E. coli* strain JM109 or XL1Blue indicating the expression of levansucrase by its own promoter. But, in *E. coli* BL21-DE3, expression of levansucrase was induced by IPTG up to 16 fold

(Table 1), suggesting the expression of levansucrase by T7 promoter in the plasmid pLS3 or pLS3105. Similarly, *sacC* gene encoding an extracellular sucrase of *Z. mobilis* was also overexpressed in *E. coli* BL21-DE3 up to 70 fold (Kannan *et al.*, 1995).

Table 1 Expression and IPTG induction of levansucrase in different *E. coli* strains

<i>E. coli</i> strain (Plasmids)	Sucrase Activity U/mg		
	LB	M63	M63+ IPTG
JM109 (pBSKS ⁺)	<0.001	<0.001	<0.001
JM109 (pLS3)	0.60	1.23	1.20
JM109 (pLS3105)	0.28	0.62	0.58
XL ₁ Blue (pBSKS ⁺)	<0.001	<0.001	<0.001
XL ₁ Blue (pLS3)	0.56	1.12	1.12
XL ₁ Blue (pLS3105)	0.26	0.60	0.60
BL21 (pBSKS ⁺)	<0.001	<0.001	<0.001
BL21 (pLS3)	1.98	4.0	72.0
BL21 (pLS3105)	0.95	2.0	20.0

Sequence analysis of the mutant of *sacB*

The nucleotide sequence of the *sacB* mutant gene is shown in **Fig. 2**. The comparison of this sequence with the already published sequence of *sacB* gene (Gunasekaran *et al.*, 1995) revealed changes at positions 304 (TTT to CTT) and 781-782 (TGG to AAG) leading to two amino acid changes, Phe-102 to Leu and Trp-261 to Lys. Similarly, replacement of Arg-331 by Lys or Leu resulted in selective elimination of levam-forming activity of *B. subtilis* levansucrase (Chambert & Petit-Glatron, 1991). But, in accordance with our earlier studies on chemical modification of levansucrase (Sangiliyandi *et al.*, 1999), mutation at the site of Cys residues should have been observed for eliminating the levam-forming activity of SacB. Though Cys residues were not affected by *in vitro* mutation,

the two mutations occurred in the vicinity of Cys residue (Fig. 2).

WILD	GGTTATTTCTATTACGTTGGTGGGAAGCAACTGGATT TTT GGTGGTCATCTTCTGAAAGAT	327
	GlyTyrPheTyrSerArgGlyGlySerAsnTrpIle Phe GlyGlyHisLeuLeuLysAsp	109
MUTANT	TGGTTATTTCTATTACGTTGGTGGGAAGCAACTGGATT CTT GGTGGTCATCTTCTGAAAGAT	327
	GlyTyrPheTyrSerArgGlyGlySerAsnTrpIle Leu GlyGlyHisLeuLeuLysAsp	109
WILD	GGTGCCAATCCGCGTTCTTGGGAATGGTCTGGT TGC ACGATTATGGCACCGGGTACGGCC	387
	GlyAlaAsnProArgSerTrpGluTrpSerGly Cys ThrIleMetAlaProGlyIleAla	129
MUTANT	TGGTGCCAATCCGCGTTCTTGGGAATGGTCTGGT TGC ACGATTATGGCACCGGGTACGGCC	387
	GlyAlaAsnProArgSerTrpGluTrpSerGly Cys ThrIleMetAlaProGlyIleAla	129
WILD	CCTGTTCCACCAAAAACCGAAACGCCTGATGGCGCTCGCTATT GTG GCTGCTGCCATTGGT	747
	ProValProProLysThrAspThrProAspGlyAlaArgTyr Cys AlaAlaAlaIleGly	249
MUTANT	TCCTGTTCCACCAAAAACCGAAACGCCTGATGGCGCTCGCTATT GTG GCTGCTGCCATTGGT	747
	ProValProProLysThrAspThrProAspGlyAlaArgTyr Cys AlaAlaAlaIleGly	249
WILD	ATTGCACAGGCCCTTAATGAAGCCCGCACCGAAT TGG AAATTGTTACCGCCTTTGGTAACC	807
	IleAlaGlnAlaLeuAsnGluAlaArgThrGlu Trp LysLeuLeuProProLeuValThr	269
MUTANT	ATTGCACAGGCCCTTAATGAAGCCCGCACCGA AAA AGAAATTGTTACCGCCTTTGGTAACC	807
	IleAlaGlnAlaLeuAsnGluAlaArgThrGlu Lys LysLeuLeuProProLeuValThr	269

Figure 2. Comparison of the nucleotide sequence of mutant *sacB* with wild type *sacB*. The nucleotide sequence number begins from the first ATG. Dark letters show the sites of mutation. The Cys residues are underlined.

In conclusion, Levansucrase (SacB) of *Z. mobilis* transfers fructosyl residue from sucrose to water (hydrolysis), mono- (or) disaccharides (or) fructo-oligosaccharides (levan-formation). The selective elimination of the levan-forming activity of SacB has been a subject of interest to us in understanding its catalytic mechanism, since SacB reduces the yield of ethanol production by *Z. mobilis*. The earlier studies on inhibition kinetics indicated the role of Cys in separately modulating the levan-forming activity of SacB (Sangiliyandi *et al.*, 1999). In SacB, three Cys residues are present at positions 121, 151 and 244 that the site of mutations (Phe-102 and Trp-261) were found in this vicinity (Cys-121 and Cys-244). This suggests the possibility of selective modulation of levan-forming activity by altering the amino acid sequence in these vicinities so that the conformation of protein is modified, which eliminates levan-formation. Accordingly, Chambert & Petit-Glatron, (1991) have also suggested by site directed mutation, the stringent steric-requirements for the levansucrase to exhibit the polymerase activity.

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RESUMO

Um levansucrase (SacB) do *Zymomonas mobilis* capaz de hidrolisar a sucrose mas não de produzir levan foi isolado completamente in vitro, do gene clonado de *sacB*. Quando o gene do mutante *sacB* foi expressado em cadeias de *Escherichia coli*, somente 50% da atividade sucrose-hydrolysing (2,0 U/mg) foi produzida, comparado ao levansucrase tipo selvagem 4,0 U/mg). Sequência do *sacB* (gene mutante) revelou mudanças de dois resíduos do amino-ácido (Phe-102 ao Leu e Trp-261 ao Lys no levansucrase). A ausência de mutação no local do Cys de SacB é contraditória à inibição do cinética que demonstrou a participação da Cys em conferir a atividade de formação do levan ao SacB. A presente descoberta é útil ao entendimento do mecanismo da modulação seletiva da formação do levan (polymerase) atividade do levansucrase.

REFERENCES

- Busby, S., Irani, M., Crombrugghe, B.D. (1982), Isolation of mutant promoters in the *Escherichia coli* galactose operon using local mutagenesis on cloned DNA fragments., *J. Mol. Biol.*, **154**, 197-209
- Chambert, R., Petit-Glatron, M.F. (1991), Polymerase and hydrolase activities of *Bacillus subtilis* levansucrase can be separately modulated by site-directed mutagenesis. *Biochem. J.*, **279**, 35-41
- Gunasekaran, P., Mukundan, G., Kannan T.R., Velmurugan, S., Ait-Abdelkader, N., Alvarez-Macarie, E, Baratti, J. (1995), The *sacB* and *sacC* genes encoding levansucrase and sucrase form a gene cluster in *Zymomonas mobilis*. *Biotechnol. Lett.*, **17**, 635-642
- Kannan, T.R., Mukundan, G., Gunasekaran, P. (1993), Fermentation characteristics of levansucrase mutants of *Zymomonas mobilis*. *J. Ferm. Bioeng.*, **75**, 265-270
- Kannan, T.R., Mukundan, A.G., Ait-Abdelkader, N., Augier-Magro, V., Baratti, J., Gunasekaran, P. (1995), Molecular cloning and characterization of the extracellular sucrase gene (*sacC*) of *Zymomonas mobilis*. *Arch. Microbiol.*, **163**, 195-204
- Lepasant, J.A., Lepasant-Kejzlarova, J., Pascal, M., Kunst, F., Billault, A., Dedonder, R. (1974), Identification of the structural gene of levansucrase in *Bacillus subtilis* Marburg. *Mol. Gen. Genet.*, **128**, 213-221
- O'Mullan, P., Szakacs-Dobozi, M., Eveleigh, D.E. (1991), Identification of saccharolytic enzymes of *Zymomonas mobilis* CP4. *Biotechnol. Lett.*, **13**, 137-142
- Sangiliyandi, G., Gunasekaran, P. (1998) A simple method for purification of thermostable levansucrase of *Zymomonas mobilis* from a recombinant *E. coli*. *J. Microbiol. Meth.*, **33**, 153-156
- Sangiliyandi, G., Chandra Raj, K., Gunasekaran, P. (1999), Elevated temperature and chemical modification selectively abolishes levan forming activity of levansucrase of *Zymomonas mobilis*. *Biotechnol. Lett.*, **21**, 179-182
- Studier, F.W., Mofatt, B.A. (1986), Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. *J. Mol. Biol.*, **189**, 113-130
- Viikari, L. (1984), Formation of levan and sorbitol from sucrose by *Zymomonas mobilis*. *Appl. Microbiol. Biotechnol.*, **19**, 252-255

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