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The effect of amphiphilic compounds on the secretion of levansucrase by *Zymomonas mobilis*

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Abstract

The effect of some aliphatic (*n*-butanol to *n*-hexadecanol) and aromatic (benzyl and phenethyl alcohols), anesthetics (procaine) and surfactants (Tween 20 to Tween 80) on the secretion of levansucrase by the levan-producing strain of Gram-negative ethanologenic bacteria *Zymomonas mobilis 113S* were examined in this study.

During incubation of *Z. mobilis* cells with sucrose (10 mM) a decrease of the levansucrase activity was observed in the presence of these amphiphilic compounds concomitantly with an increase of a total amount of protein in the medium. Since none of the compounds under study had any effect on enzyme activity in vitro observed structure- and concentration-dependent relationships most probably reflected differently conditioned processes of membrane-associated secretion of levansucrase and total protein by *Z. mobilis*. The patterns of fluorescence titrations by ANS^- indicated to competitive interactions between an amphiphilic compound of varied structure and the probe for the polar and non-polar binding sites of *Z. mobilis* membrane structures. The effect of 2,4-DNP (protonophore) and sodium azide (an inhibitor of ATPase) alone as well as in combination with aliphatic alcohols suggested to the participation of energy transduction system in the secretion of levansucrase by *Z. mobilis* cells. Under conditions of abolished proton motive force (PMF) the level of levansucrase decreased whereas the amount of protein elevated significantly in the medium in accordance with the expected requirement of PMF to perform the secretion of levansucrase and to keep intact the permeability barrier of cells.

Keywords: Protein secretion; Levansucrase; Amphiphilic compound; Permeability; Membranes; *Zymomonas mobilis*

1. Introduction

Extracellular levansucrases (EC 2.4.1.10) of various bacteria catalyse the synthesis of levan, the β-2,6-linked fructose polymer [1,2]. This polysaccharide possesses good prospects for several biomedical applications and Gram-negative ethanologenic bacterium *Zymomonas mobilis* exibit strong potential for its biosynthesis in sucrose-containing media [1–4]. However, levan as a by-product diminishes the efficiency of ethanol production in *Z. mobilis* [1,5,6] and from this standpoint levan formation should be reduced to improve the ethanol yield on sucrose-containing substrates [6]. The accumulation of levan in the medium obviously can be influenced by the amount of secreted levansucrase. Hence, an elucidation of potent factors to affect levansucrase secretion would give an appropriate tool to modify the yield of products as well as to specify processes of enzyme secretion in *Z. mobilis* which remain scarcely investigated at present [7–10].

Increasing amount of evidence suggest an essential role of lipid–protein interactions to govern the secretion of exoproteins across membranes, including the Sec system or general secretory pathway (GSP) for Gram-negative bacteria [11,12].

A wide variety of chemicals (aromatics, xenobiotics, alcohols, alkanes, etc.) are generally acknowledged as essential environmental factors to induce complex of structural and functional changes which are related to the ability of a compound for partition into the cytoplasmic membrane of bacteria [13,14]. These substances are mostly amphiphilic, i.e. each contains both hydrophilic and hydrophobic region in the molecule thus reproducing similar properties of membrane

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glycerophospholipids and proteins [13]. The affinity of the amphiphilic compound for bacterial membranes therefore depends on the molecular size and the degree of hydrophobicity. Thus, aliphatic alcohols, containing polar hydroxyl groups are increasingly hydrophobic in proportion to nonpolar alkyl chain length [13–16]. A lot of physiological responses have been found to disturbance of membrane structure by amphiphilic compounds, including alterations in secretion of levansucrase by *Bacillus subtilis* [17] as well as other exoenzymes by various species of this genera [18] whereas Gram-negative bacteria remain uninvestigated in this respect.

It should be noted that overall process of protein secretion in *Z. mobilis* still remain obscure, except reported gradual specifications on glucose-fructose oxidoreductase (EC 1.1.1.99) as a TAT-dependent process [7,8], statements on the absence of N-terminal signal peptide and enhanced secretion of levansucrase due to an interaction between the specific intracellular protein and the cell outer membrane [9,10]. This study represents an attempt to examine the effects of some aliphatic and aromatic alcohols, anesthetics and surfactants on the secretion of levansucrase and total protein into the medium by the levan-producing strain *Z. mobilis 113S* in respect of structure and concentration of these amphiphilic compounds.

2. Materials and methods

2.1. Strain and culture conditions

The *Z. mobilis 113S* strain [19] was maintained and cultivated batchwise without aeration for 8 h (late logarithmic phase) at 30 °C, as reported previously [20], except that the fermentation medium contained 0.5% yeast extract and 10% glucose as a sole carbon source.

2.2. Preparation of Cells and incubation conditions

The cells form the late logarithmic phase of growth ($\mu = 0.150–0.20\ h^{-1}$) were recovered by centrifugation (5000 × *g*, 15 min, 20 °C) and washed twice with 50 mM Tris–malate buffer (pH 5.3). Incubations were performed at the same buffer containing sucrose (10 mM), cells (2.0–2.5 mg dry biomass × ml^{-1}) in the absence (controls) and the presence of compounds under study (0.04–10 mM) for 0.5 h at 30 °C. Cells were separated by centrifugation (7000 × *g*, 10 min) and supernatant stored on ice for subsequent assays.

Aliphatic alcohols (1-butanol up to 1- hexadecanol), benzyl alcohol, phenethyl alcohol and procaine hydrochloride were from Sigma-Aldrich Chemie GmbH (Germany), Tween 20 up to Tween 80 from Ferak Laborat. GmbH (Germany). To ensure homogeneity of incubation medium for hydrophobic compounds stock solutions were prepared in purified dimethyl formamide (DMFA). Additions of DMFA to the incubation medium did not exceed 0.8% (v/v), including controls.

2.3. Enzyme assay

The extracellular levansucrase (EC 2.4.1.10) in the incubation medium was assayed spectrophotometrically (UV 260, Shimadzu, Japan) by measuring the initial velocity of levan formation at 400 nm ($\varepsilon_{400} = 3.06 \times 10^5$ L (mol cm)$^{-1}$) as described previously [21] and expressed as the activity units (U) per mg dry mass of cells in the incubation medium.

2.4. Fluorescence titration measurements

The effects of amphiphilic compounds on the binding of increasing amount (5–60 μM) of the fluorescent probe ANS^- (1-anilinonaphtalene-8-sulfonate) by *Z. mobilis 113S* cells (3.0 mg ml^{-1} cells in 50 mM Tris–malate buffer pH 5.3) were determined on a Fluorichrom (Varian, Inc., CA, US) fluorimeter equipped by Coach (Couch Suplex MA, US) software at 380 nm for exitation and 500 nm for emission. Titration results were expressed as differences between fluorescence signals (arbitrary units) of ANS^- from treated and untreated cells.

2.5. Other analytical determinations

The biomass concentration was determined spectrophotometrically at 550 nm using the calibration curve as described previously [22]. Protein content in the incubation medium was assayed by the dye-binding (Coomassie Brilliant Blue G250, Sigma) Lowry (Folin-Ciocalteu's reagent, Fluka Chem. AG, Switzerland) procedures [23,24], which gave compatible results, except observed significant dye-binding by surfactants [23]. Ethanol concentration was analysed by routine procedures as described elsewhere [20] and levan concentration assayed with the resorcinol method after precipitation by ethanol (2.6:1, v/v) from the incubation medium [25].

2.6. Data processing and analysis

Factor analysis was performed accordingly to 2^2- and 3^2-design of experiments and processed by ANOVA, pair and multiple non-linear regression using software Statgraphics® Plus (Manugistics, Inc., US) and Mathcad (Cambridge Mass., US), confidence levels of treatment effects were evaluated by the *F*-test.

Incubations were performed at least at triplicate and data in the figures are given as the mean ± S.E. estimated by ANOVA.

3. Results

During incubation of *Z. mobilis* cells the activity of levansucrase and the total amount of secreted protein in the medium were markedly affected in the presence of aliphatic alcohols (Figs. 1–4). However, observed effects were