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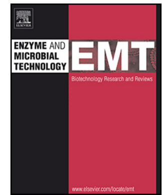

Improvement of catalytical properties of two invertases highly tolerant to sucrose after expression in *Pichia pastoris*. Effect of glycosylation on enzyme properties

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ABSTRACT

Zymomonas mobilis genes encoding INVA and INVB were expressed in *Pichia pastoris*, under the control of the strong AOX1 promoter, and the recombinant enzymes were named $INVA_{AOX1}$ and $INVB_{AOX1}$. The expression levels of $INVA_{AOX1}$ (1660 U/mg) and $INVB_{AOX1}$ (1993 U/mg) in *P. pastoris* were 9- and 7-fold higher than those observed for the native INVA and INVB proteins in *Z. mobilis*. $INVA_{AOX1}$ and $INVB_{AOX1}$ displayed a 2- to 3-fold higher substrate affinity, and a 2- to 200-fold higher catalytic efficiency (k_{cat}/K_M) than that observed for native INVA and INVB from *Z. mobilis*. Positive Schiff staining of $INVA_{AOX1}$ and $INVB_{AOX1}$ suggested a glycoprotein nature of both invertases. After deglycosylation of these enzymes, denoted D-$INVA_{AOX1}$ and D-$INVB_{AOX1}$, they exhibited a 1.3- and 3-fold lower catalytic efficiency (107 and 164 s^{-1} mM^{-1}, respectively), and a 1.3- to 5-fold lower thermal stability than the glycosylated forms at temperatures of 35–45 °C. After deglycosylation no effect was observed in optimal pH, being of 5.5 for $INVA_{AOX1}$, $INVB_{AOX1}$, D-$INVA_{AOX1}$ and D-$INVB_{AOX1}$. The invertase activity of both enzymes increased in 80% ($INVA_{AOX1}$) and 20% ($INVB_{AOX1}$) in the presence of Mn^{2+} at 1 mM and 5 mM, respectively. $INVA_{AOX1}$ and $INVB_{AOX1}$ were highly active at sucrose concentrations of up to 400 and 300 mM, respectively; however, the tolerance to sucrose decreased to 300 mM for D-$INVA_{AOX1}$. Our findings suggest that glycosylation of $INVA_{AOX1}$ and $INVB_{AOX1}$ plays an important role in their thermal stability, catalytic efficiency, and tolerance to sucrose. In conclusion, the expression of INVA and INVB from *Z. mobilis* in *P. pastoris* yields new catalysts with improved catalytic properties, making them suitable candidates for a number of industrial applications or for the improvement of ethanol production from cane molasses.

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1. Introduction

For several years, three types of glycosyl hydrolases have been studied in *Zymomonas mobilis* [1]. Two of them belong to the invertase or β-D-fructofuranosidase group (EC.3.2.1.26), which are involved in catalyzing the release of D-glucose and D-fructose in an equimolar mixture from non-reducing ends of sucrose or β-D-fructofuranoside [2,3]. These none-crystal-forming sugars are important in the food industry, primarily because they can be used in confectionery in soft-centered chocolates and as sweetener (fructose) for diabetics [4]. Currently, *Aureobasidium* spp., *Rhodotorula glutinis*, *Saccharomyces cerevisiae*, and *Saccharomyces carlsbergensis* are the main yeast species that produce commercial invertases [4], and so far, production of commercial invertases from bacteria has not been reported.

The invertases from *Z. mobilis* have been intensively studied. Different research groups have cloned the intracellular invertase A (INVA) in *Escherichia coli* [5–7], sequenced [5], purified, characterized [5–7,11], and immobilized on Avicel [11]. The extracellular invertase B (INVB) was first characterized and purified by Preziosi et al. [1] and O'Mullan et al. [8]. Then, this enzyme was cloned in *E. coli*, characterized and sequenced [9–11], expressed, produced [12–15] and immobilized on Avicel [14] and Nylon-6 [15], using *E. coli* as bacterial expression system. An advantage of these

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Table 1
Invertases heterologous expressed in *Pichia pastoris*/AOX1 promoter.

Organism enzyme	Vector	MW (kDa)	K_M (mM)	Optimum pH/temp (°C)	Ions activator/inhibitor	Specific activity (U/mg)	Ref.
S. cerevisiae SUC2	pGS102 AOX1	85–90 (G)	Nd	Nd	Nd	3000	[20]
S. cerevisiae SUC2	Nd	85 (G)	Nd	6.0, 55	Nd	3400	[21]
I. batatas L. Ibβfruct1	pPICZαB AOX1	72 (G)	10.69	5.0, 40	Nd	1.08	[22]
B. vulgaris L. 6-FEH	pPICZαA AOX1	75	77	5.0, 30	Nd	12.5	[23]
I. batatas L. Ibβfruct2 Ibβfruct3	pPICZαC AOX1	82 (G) 87 (G)	4.97 10.1	5.0 5.0	Nd	553 70	[24]
B. oldhamii Boβfruct2 Boβfruct3	pPICZαB AOX1	77.5 (G) 77.5 (G)	0.42 22.9	3.0, 60 4.0, 50	Ca^{2+},Co^{2+}/Hg^{2+} None/Hg^{2+}	884.3 1694.5	[25]
Allium cepa GFT-INV INV	pPICZαC AOX1	Nd	1 6	Nd	Nd	45 4.3	[26]

G—glycosylated; Nd—not determined.

enzymes is their high tolerance to sucrose (300–400 mM) compared to the commercial invertase from *S. cerevisiae* (150 mM) [16,44]. However, the expression levels of both INVA and INVB in *Z. mobilis* are very low [8,10], limiting their use at the industrial scale.

The *Pichia pastoris* expression system has been widely used as an alternative way to increase the expression levels of target proteins, because it is very efficient at highly expressing heterologous genes when it grows in the presence of methanol as an energy source [17,19,28]. Additionally, the expression of proteins of interest in *P. pastoris* can be scaled to higher levels than those achieved with *E. coli*, and in many cases, it is also superior to those achieved with *S. cerevisiae*. For this reason, more than 1000 proteins have been expressed in *P. pastoris* over the past three decades [17–19,28].

Currently, there are several studies on invertase expression under the control of the AOX1 promoter in *P. pastoris* (Table 1). Invertases from *Bambusa oldhammi* [25], *Ipomoea batatas* L. [22,24], *Beta vulgaris* L. [23], *Allium cepa* [26] and invertase Suc2 from *S. cerevisiae* [20,21], among others, have been successfully expressed in *P. pastoris* under the control of the AOX1 promoter, and characterized. Most recombinant invertases are glycosylated, because O- and N- linked glycosylation is the most common post-translational modification performed by this host [17,27,28].

The aim of this work was to express and characterize the INVA and INVB invertases from *Z. mobilis* in *P. pastoris*, under the control of the AOX1 promoter, and to evaluate the effect of glycosylation on the resulting recombinant proteins.

2. Materials and methods

2.1. Strains, plasmids, and culture media

A *Z. mobilis* CDBB-B603 strain was obtained from the "Colección de Cultivos Microbianos" (CINVESTAV-IPN, México) and was grown as described by Bekers et al. [29]. *E. coli* DH5α (Novogen, USA) cells were used for vector propagation and construction. *P. pastoris* X-33 and pPICZαB (Invitrogen, Carlsbad, CA, USA) were used as host and vector, respectively, for heterologous expression, according to the manufacturer's instructions. *E. coli* DH5α was grown at 37 °C with shaking at 250 rpm for 16–18 h in LB medium supplemented with 25 μg/mL Zeocin (Invitrogen).

Yeast cultures were grown at 30 °C with agitation at 200 rpm for 3–5 days in YPD or YPDS medium. *P. pastoris* transformants were selected on 2,3,5-triphenyltetrazolium chloride (TTC)-indicator plates according to the method of Bochner and Savageau [32] with slight modifications, MM medium with 1% (v/v) methanol, MD with 1% (w/v) dextrose, BMGY with 1% (v/v) glycerol supplemented with 100 μg/mL Zeocin when necessary, and BMMY with 1% (v/v) methanol to achieve enzyme induction [30].

Table 2
List of oligonucleotide primers used for PCR studies in this work.

Name	Sequence (5′–3′)
INVA-forward (AF)	GAA CTG CAG GTG AAT CCC CCT CTT ATA AAA ATT TA TC
INVA-reverse (AR)	TGC TCT AGA TTA ACA GGC ATC GCT TGA AAA AGC G
INVB-forward (BF)	GAA CTG CAG GGT TTA ATT TTA ATG CCA GTC GCT GG
INVB-reverse (BR)	TGC TCT AGA TTA TTT GCG ACG ATC AGG GAA AGG CC
α-Factor forward	TAC TAT TGC CAG CAT TGC TGC
3′ AOX reverse	GCA AAT GGC ATT CTG ACA TCC

2.2. Subcloning of the invertase *invA* and *invB* genes in pPICZαB

The YeaStar™ Genomic DNA Kit (Zymo Research, Irvine, CA, USA) was used to isolate *Z. mobilis* DNA and to amplify the ORF of the *invA* and *invB* genes (without initial methionine) by PCR, using *Pfu* DNA Polymerase (Fermentas, Germany) and the primers listed in Table 2. INVA-F and INVA-R primers were used for the amplification of the ORF *invA*, while the ORF *invB* was amplified using the primers INVB-F and INVB-R. The forward primers INVA-F and INVB-F included a PstI restriction site (underlined), while the reverse primers INVA-R and INVB-R contained an XbaI restriction site (underlined) and a stop codon (bold), to avoid the translation of the C-terminal peptide, including the c-myc epitope and polyhistidine tag.

The PCR products were purified using the QIAquick® Gel Extraction Kit (Qiagen, Germany) and cloned into the PstI and XbaI restriction sites of the pPICZαB vector (Invitrogen) to generate the constructs pPICZαB-*invA* and pPICZαB-*invB*, which were used to transform *E. coli* DH5α cells by electroporation. Positive clones were selected by screening for Zeocin resistance (100 μg/mL).

2.3. Invertase and protein assays

Invertase activity was determined by measuring the release of reducing sugars using 3,5-dinitrosalicylic acid (DNS) as described by Miller [31]. The assay mixture contained 0.05 M sucrose in 0.05 M acetate buffer at pH 5.0 (INVA) or 5.5 (INVB), and enzyme preparations were incubated at 35 °C for INVA and 40 °C for INVB. The reducing sugars that formed after incubation were quantified at 540 nm. The enzymatic activity was expressed in U/mL, with one

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