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High level production of a recombinant acid stable exoinulinase from *Aspergillus kawachii*

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ABSTRACT

Exoinulinases—enzymes extensively studied in recent decades because of their industrial applications—need to be produced in suitable quantities in order to meet production demands. We describe here the production of an acid-stable recombinant inulinase from *Aspergillus kawachii* in the *Pichia pastoris* system and the recombinant enzyme's biochemical characteristics and potential application to industrial processes. After an appropriate cloning strategy, this genetically engineered inulinase was successfully overproduced in fed-batch fermentations, reaching up to 840 U/ml after a 72-h cultivation. The protein, purified to homogeneity by chromatographic techniques, was obtained at a 42% yield. The following biochemical characteristics were determined: the enzyme had an optimal pH of 3, was stable for at least 3 h at 55 °C, and was inhibited in catalytic activity almost completely by Hg^{+2}. The respective K_m and V_{max} for the recombinant inulinase with inulin as substrate were 1.35 mM and 2673 μmol/min/mg. The recombinant enzyme is an exoinulinase but also possesses synthetic activity (*i. e.*, fructosyl transferase). The high level of production of this recombinant plus its relevant biochemical properties would argue that the process presented here is a possible recourse for industrial applications in carbohydrate processing.

1. Introduction

Inulinases—as the name suggests—are enzymes that degrade inulin, a member of the fructans family and a plant energy-storage polymer composed of variable-length linear fructose chains ending in linkage to a β-2-glucose residue [1,2]. According to their mode of catalysis, the inulinases are classified into two types: the endoinulinases that hydrolyze internal 1,2-fructofuranosidic bonds and generate short fructololigosaccharides and the exoinulinases that hydrolyze the polymer from the terminus and release fructose units [3]. Microbial inulinases have many applications in industrial processes such as the production high-fructose syrup via complete inulin hydrolysis, or the synthesis of fructololigosaccharides through the use of highly concentrated solutions of sucrose [4,5]. Fructose is a sweetener of the Food and Drug Administration category Generally Recognized as Safe (GRAS), thus considered as a healthy alternative to sucrose since the monomer possesses a greater sweetening capacity and is furthermore beneficial to diabetic patients [6]. Industrial conditions for the chemical hydrolysis of inulin (*i. e.*, high temperatures, 80–100 °C and acid pHs between 1 and 2), result in product degradation and certain by-products are obtained, such as difructose anhydride, that change the properties of the final product to a dark brown solution with a lower sweetening capability [7]. Nevertheless, fructololigosaccharides have gained attention as functional-food components because of the associated bifidogenic and health-promoting properties [8,9].

Many microorganisms are reported to produce inulinases such as bacteria, yeast, and other fungi. The filamentous fungus *Aspergillus kawachii*, traditionally used in the Japanese alcoholic-beverage industry, produces several enzymes with acid-depolymerase activities such as amylases, xylanases, and proteinases [10–12]. In our laboratory, we found that *A. kawachii* produced, among other enzymes, an exoinulinase with promising properties for a potential use in the food industry [13].

The production of enzymes with potential industrial application through the use of wild microorganisms such as bacteria, yeast, or other fungi is sometimes not feasible because of low productivity levels or the costs associated with a large-scale purification. These considerations indicate the relevance of producing those classes of enzyme in

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heterologous systems, such as *Saccharomyces cerevisiae* and *Pichia pastoris,* in a process having a potential industrial application [14,15].

Pichia pastoris is a eukaryotic microorganism widely used as a heterologous-expression system. In addition to the yeast's productive advantages, such as high culture densities and yields of the recombinant enzyme, an efficient secretion system would point to that microorganism as being an appropriate system to overexpress proteins [16,17].

Although several reports are available about specific aspects of the cloning and overexpression of these kinds of enzymes, we present here the whole procedure—*i. e*, from the initial cloning to the final application of this enzyme to industrial processes—with detailed information about the genetic engineering involved. The specific system under study was the production of a recombinant exoinulinase gene from *A. kawachii* that became overexpressed in *P. pastoris* and possessed a potential for industrial applications. We describe here the successful production, purification, and characterization of the recombinant enzyme and discuss those possible industrial uses.

2. Materials and methods

2.1. Chemicals

Inulin was obtained from Sigma-Aldrich (St Louis, MO, USA); Promega (Madison, WI, USA) provided the enzymes used—namely, the restriction endonucleases (*Xho*I, *Not*I, *Bgl*II, *Avr*II), Taq DNA polymerase, calf intestinal alkaline phosphatase, T4 DNA ligase—along with the cloning and expression systems such as the pGEM™-T Easy Vector, the Wizard SV Gel, and the clean-up kit for the polymerase-chain reaction (PCR). The IllustraTM MicroSpin columns and DNA-purification kit were from GE Healthcare (Fairfield, CT, USA) and the *BsrD*I restriction endonuclease from New England Biolabs (Beverly, MA, USA). Each of these products was used as indicated by the manufacturer. All the other reagents used were of analytical grade.

2.2. Strains, plasmids, and culture conditions

Aspergillus kawachii IFO 4308 strain was kindly provided by the Institute for Fermentation in Osaka, Japan and used as the source of the DNA.

The microbial strains (*Escherichia coli* TOP10F′, *P. pastoris* GS115 and X33) and the plasmids (pPIC9 and pPICZαA) were acquired from Invitrogen Co. Ltd., (Carlsbad, CA, USA).

For the growth of *A. kawachii* cells, the culture medium previously described by Contreras Esquivel and Voget [18], and in the present work containing 10 g/l of inulin as the carbon and energy source, was inoculated with 10^6 spores/ml of the fungus. The cultures were then incubated at 28 °C and 200 rpm for 48 h before harvesting.

Recombinant *E. coli* TOP10F′ cells were grown in Luria-Bertani medium with 0.1 mg/ml of ampicillin or 0.025 mg/ml Zeocin™ when necessary.

Pichia pastoris GS115 and X-33 cells were cultured in medium containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose (YPD) and supplemented with 0.1 mg/ml of Zeocin™. As suggested by the manufacturer for *P. pastoris* proliferation, the buffered–glycerol-complex medium BMGY was used; whereas, for the induction of the alcohol-oxidase promoter AOX, the buffered–methanol-complex medium BMMY was used. The composition of BMGY was 20 g/l peptone, 13.4 g/l yeast-nitrogen base, 10 g/l glycerol, 10 g/l yeast extract, and 0.4 g/l biotin. The BMMY medium composition was equivalent to that of BMGY but with the glycerol being replaced by 5 g/l methanol. *Pichia pastoris* X-33 transformants were grown on minimal dextrose medium containing 20 g/l agar, 20 g/l dextrose, 13.4 g/l yeast-nitrogen base, and 0.4 mg/l. For cultures performed in the bioreactor (as batch and fed-batch fermentation) basal salt medium BSM (40 g/l glycerol, 6.40 g/l K_2SO_4, 1.80 g/l KOH, 3.40 g/l $MgSO_4.7H_2O$, 0.36 g/l $CaSO_4.2H_2O$, 12 ml/l H_3PO_4) was used supplemented with the *P. pastoris*–growth-promoting salt solution PTM1 (4 ml/l) plus 8 ml/l of a D-biotin solution (0.20 g/l). PTM1 contained 0.5 g/l $CoCl_2.6H_2O$, 2.0 g/l $CuSO_4$, 22.0 g/l $FeSO_4.7H_2O$, 5 ml H_2SO_4, 0.02 g/l H_3BO_3, 0.08 g/l KI, 3.0 g/l $MnSO_4$, 0.2 g/l Na_2MoO_4, 7.0 g/l and $ZnCl_2$.

2.3. Genomic-DNA extraction and the PCR for the amplification of the inulinase gene

The extraction of genomic DNA from *A. kawachii* was performed following a previously described method [19].

The design of proper primers for cloning the *inuAk* gene was based on previous information on the N-terminal-amino-acid sequence of the *Aspergillus awamori* inulinase (fw primer) and on a consensus C-terminal-amino-acid sequence of related enzymes; *i. e.*, the A*spergillus foetidus* fructosyltransferase and the inulinases A and B from *Aspergillus niger* [rv primer] [20].

Genomic DNA was amplified by PCR with the upstream primer P1: 5-TAGTATCT*CTCGAG*AAGAGAGCTCCTCTGTCGAAG-3 (with an added *Xho*I site indicated in italics) and the downstream primer P2: 5-TACT CT*CCTAGG*TCAATTCCACGTCGAAG-3 (with the sequence in italics being the position of an *Avr*II site). The amplification was carried out in a Mastercycler (Eppendorf AG, Hamburg, Germany) with Taq DNA polymerase under the following conditions: 95 °C for 180 s, 30 cycles at 95 °C for 60 s, 56 °C for 30 s, and 72 °C for 60 s, with a consolidation step at 72 °C for 300 s. The PCR product was purified by the DNA-purification kit containing Illustra™ MicroSpin Columns.

Both the amplicon-containing the inulinase gene and the empty pPic9 vector were digested with *Xho*I plus *Avr*II. Calf-intestine alkaline phosphatase was used to dephosphorylate the pPIC9 plasmid so as to prevent self-ligation. The inulinase gene was ligated to pPIC9 with T4 DNA ligase to obtain the recombinant vector pPIC9:*inulinase*. The correct sequence of the open-reading frame (ORF) was confirmed by sequence analysis (Macrogen Inc., Gasan-dong, Seoul, 153-023, Republic of Korea).

2.4. Intron and signal-peptide excision from the inuAk gene

Information in the literature on the inulinase genes was used to design the cloning strategy. We found that the inulinase gene from *A. awamori* was composed of two exons and a 57-bp intron [21]. We then confirmed this information bioinformatically *in silico* and compared the structure with that of the *A. kawachii* genes. From this analysis, we also noticed a possible signal peptide in the sequence. To construct the posttrancriptional region of the *A. kawachii* gene after splicing, both the signal peptide and the intron were excised through the use of PCR and enzymatic restriction.

In order to obtain exon I, a PCR reaction was performed by means of the plasmid vector pPIC9:*inulinase* as a template and P3 and P4 as primers. Primer P3 5′-TGCAGTCG*CTCGAG*AAGAGATTCAACTATGACC AGCCT-3′, with the sequence in italics being a *Xho*I restriction site—was designed to remove the signal peptide. Primer P4 5′-ACGCG*G CAATG*TTTGCGCAACGGGGTAATAGGAAGTATACATGGCG-3′ was designed to span the intron, with the first 19 nucleotides overlapping the 3′end of exon I and the following 16 nucleotides overlapping the 5′ end of exon II, including the recognition site of *BsrD*I (in italics). The reaction was performed with 30 cycles at 94 °C for 60 s, 56 °C for 60 s, and 72 °C for 60 s. The PCR product (383 bp) was gel-purified through the use of a DNA-purification kit Illustra™ MicroSpin Columns, ligated to pGEM™–T Easy Vector and transformed into *E. coli* TOP10F'. The recombinant vectors isolated from the positive transformants were digested with *Xho*I and *BsrD*I.

In order to obtain the second exon, the pPic9:*inulinase* construction was digested with *BsrD*I and *Not*I to release exon II, and that DNA fragment of 1200 bp was then purified by agarose-gel electrophoresis.

Finally, both exons were ligated to pPicZαA (previously digested by