Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 51 (2007) 110–119
Protein Expression & Purification
www.elsevier.com/locate/yprep

High level production of the *Magnaporthe grisea* fructose 1,6-bisphosphate aldolase enzyme in *Escherichia coli* using a small volume bench-top fermentor

Geneviève Labbé, Jeremy Bezaire, Sarah de Groot, Christine How, Tim Rasmusson, Jason Yaeck, Eric Jervis, Gary I. Dmitrienko, J. Guy Guillemette *

Department of Chemistry, University of Waterloo, 200 University Ave. W, Waterloo, Ont., Canada N2L 3G1

Received 18 May 2006
Available online 28 June 2006

Abstract

The Class II fructose 1,6-bisphosphate aldolase from the Rice Blast causative agent *Magnaporthe grisea* was subcloned in the *Escherichia coli* vector pT7-7. The enzyme was overexpressed using fed-batch fermentation in a small bench-top reactor. A total of 275 g of cells and 1.3 g of highly purified enzyme with a specific activity of 70 U/mg were obtained from a 1.5 L culture. The purified enzyme is a homodimer of 39.6 kDa subunits with a zinc ion at the active site. Kinetic characterization indicates that the enzyme has a K_m of 51 μM, a k_{cat} of 46 s^{-1}, and a pH optimum of 7.8 for fructose 1,6-bisphosphate cleavage. The fermentation system procedure reported exemplifies the potential of using a lab-scale bioreactor for the large scale production of recombinant enzymes.

Keywords: Zinc metalloenzyme; Fed-batch fermentation; Fructose 1,6-bisphosphate aldolase; Recombinant enzymes; *Magnaporthe grisea*

Fructose 1,6-bisphosphate aldolases (FBA,[1] E.C. 4.1.2.13) catalyze the reversible aldol condensation of dihydroxyacetonephosphate (DHAP) and glyceraldehyde 3-phosphate (G3P) in the glycolysis, gluconeogenesis, and in the Calvin cycle. The enzymes are divided into two evolutionary convergent groups depending on the reaction mechanism [1]. The Class I FBAs form a Schiff-base intermediate between the carbonyl substrate (FBP or DHAP) and the active site lysine residue and are present in animals, plants, algae, and in certain prokaryotes [2]. The Class II FBAs are metal-dependent, as they require a divalent metal ion to stabilize the carbanion intermediate during catalysis. They are absent from animals and higher plants, but are found in lower organisms such as bacteria, fungi, algae, and protozoa, some of which also having Class I aldolase [3,4]. The Class II aldolases can be further divided into two groups depending on their sequence: the type A enzymes, comprising the fungal FBAs, and type B enzymes, which comprises tagatose 1,6-bisphosphate aldolases as well as FBAs [5].

It has been suggested that Class II FBA could be a viable drug target, since it is absent from animals [6,7]. Class II FBA has been found to be an essential enzyme in a number of lower organisms, including *Saccharomyces cerevisiae*, *Escherichia coli*, and *Bacillus subtilis* [8–10]. Attempts to isolate a *fba* knock-out mutant were not successful in *Streptomyces galbus* [11]. The Class II FBA is overexpressed in bacteria under stressful conditions [12–17]. *M. grisea* is among the best studied species of phytopathogenic fungi: its genome sequence as well as extensive EST/cDNA libraries are now publicly available [18–20]. The *fba* gene of *M. grisea* is also expressed at a significantly higher level

* Corresponding author. Fax: +1 519 746 0435.
E-mail address: jguillem@uwaterloo.ca (J. Guy Guillemette).

[1] *Abbreviations used:* DHAP, dihydroxyacetonephosphate; FBA, fructose 1,6-bisphosphate aldolase; FBP, fructose 1,6-bisphosphate; G3P, glyceraldehyde 3-phosphate; PAR, 4-(2-pyridylazo)-resorcinol.

1046-5928/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2006.06.020

under nitrogen starvation conditions based on comparative analysis in EST databases (the aldolase has the Unigene ID DNMag0118 in the Consortium for the Functional Genomics of Microbial Eukaryotes database maintained by the University of Exeter, UK). Thus, the Class II FBA may present a promising target for the development of new inhibitors against *M. grisea*.

Traditional culturing methods typically use batch fermentations which contain an initial amount of substrate and are harvested upon substrate exhaustion. Shake flasks are limited in both oxygen transfer and maximum attainable cell concentration due to the poor mixing and limited substrate feeding capacity. The maximum reported amount of *E. coli* FBA produced per liter of culture in shake flasks in an overexpression strain is ~150 mg, or 3500 U of the enzyme (specific activity = 23.3 U/mg) [21]. Clearly, improvements on batch cultures grown in shake flasks can be obtained by growing cultures in stirred tank bioreactors. Batch cultures grown in bioreactors facilitate oxygen transfer rates, which frequently improve cell and recombinant protein yields. The maximum cell concentration of a batch culture is typically limited by osmotic effects and the accumulation of toxic metabolic by-products at high initial substrate concentrations. Fed-batch culturing systems operated in stirred tank bioreactors allow for controlled feeding of substrate following the exhaustion of the initial batch concentration. Fed-batch fermentations of *E. coli* can reach densities as high as 183 g/L [22] on a dry cell weight basis. Volumetric product yields are also greatly improved at high cell concentrations [23]. Along with increased volumetric productivity, fed-batch culture can improve plasmid stability with respect to batch [24] and continuous [25] cultures, facilitate growth rate control, and minimize inhibitory metabolic by-product formation.

A high yield fermentation procedure was optimized to produce recombinant enzyme for kinetic characterization, inhibitor testing, and crystallographic studies. For overexpression, we subcloned the *fba* gene from a *M. grisea* BAC vector and inserted the amplified DNA into the pT7-7 *E. coli* vector. The native enzyme was produced as N-terminal affinity tags yield inactive aldolase [11,26]. Here, we demonstrate that the gene *fba* codes for the active Class II FBA, as predicted by sequence analysis. Further, the enzyme has a dimeric structure, requires Zn^{2+} for catalysis and has a specific activity of 70 U/mg. The fed-batch fermentation process yielded 211,000 U of enzyme per liter of culture; ~0.88 g of greater than 98% pure recombinant *M. grisea* aldolase could be obtained per liter of *E. coli* culture following induction for 3.5 h.

Materials and methods

All chemicals used are of analytical grade and were purchased from Sigma (St. Louis, MS) unless stated otherwise. All buffers and solutions were prepared with Milli Q water (Millipore, Bedford, MA).

Bacterial strains

The *E. coli* strain Bl21(DE3) was obtained from Novagen (Madison, WI). The *E. coli* strain XL1 Blue was obtained from Clontech (Palo Alto, CA). The subcloning efficiency DH5α competent cells were obtained from Invitrogen Canada Inc. (Burlington, ON).

Molecular cloning of the Class II FBA from M. grisea

The cDNA of *M. grisea* 70-15 containing the gene of Class II FBA is publicly available as a result of the *Magnaporthe* Sequencing Project [20]. We obtained the BAC vector containing the aldolase gene (Clone Name 20B24) from the Fungal Genetics Stock Center (School of Biological Sciences, University of Missouri, Kansas City, Missouri, USA). The aldolase has been given the GenBank protein Accession No. XP_369021. The 1805 bp sequence identified as a probable Class II FBP aldolase gene (also identified as hypothetical protein MG00223.4 in the Broad Institute website), constitutes of five exons (respectively, 54, 131, 421, 229, and 302 bp) separated by four introns (406, 85, 116, and 61 bp). We determined that the exon containing the first 18 amino acids of the GenBank sequence is actually not part of the protein (see discussion), so it was not amplified. The primers used for the amplification of the last four exons and three introns of the aldolase sequence from the BAC vector, (primers starting from position 6741 and ending at position 8153 the GenBank sequence no. AACU01001388), were: MGRISFOR1: 5′-ACGCATACTACCTTTTAAACAGG GCATATGGGTGTTCTTCAGCGAGCTCGGTCTCA AGC-3′ (with the introduced *Nde*I site underlined, corresponding to the methionine residue at the start of the second identified exon of the sequence) and MGRISREV2: 5′-CATCATTACGAGCATCAATCGATAGGTTGCGT GGGAAT**TTA**GATGGTG-3′ (with the introduced *Cla*I restriction site underlined, and the stop codon shown in bold). All primers were obtained from Sigma–Aldrich (Oakville, ON). The PCR was performed using the PWO DNA polymerase kit (Roche, Laval, QC) in a TechGene Thermal Cycler model FTgene2D (Techne, Burlington, NJ). The PCR product was recovered from a 1% agarose gel with the QIAquick gel extraction kit (Qiagen, Mississauga, ON).

We then cloned the gene in the *E. coli* expression vector pT7-7 [27]. Both vector and insert were digested with *Nde*I and *Cla*I, the digested vector was dephosphorylated with alkaline phosphatase, and the two fragments were ligated with T4 DNA ligase. The ligation mixture was used to transform *E. coli* XL1-Blue cells by electroporation. The plasmid DNA was purified using the FlexiPrep™ kit (Amersham Pharmacia Biotech, Piscataway, NJ). The resulting pT7-7/MGFBA construct was sequenced at the MOBIX facility at McMaster University (Hamilton, ON).