Protein Expression and Purification 74 (2010) 196–203

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Heterologous expression and functional characterization of a plant alkaline phytase in *Pichia pastoris*

Steven C. Johnson, Mimi Yang, Pushpalatha P.N. Murthy *

Department of Chemistry, Michigan Technological University, 1400 Townsend Drive, Houghton, MI 49931, USA

ARTICLE INFO

Article history:
Received 26 April 2010
and in revised form 16 July 2010
Available online 22 July 2010

Keywords:
Alkaline phytase
Phosphate contamination
Animal feed
Heterologous protein expression
Pichia pastoris
Pollen grains

ABSTRACT

Phytases catalyze the sequential hydrolysis of phytic acid (*myo*-insositol hexakisphosphate), the most abundant inositol phosphate in cells. Phytic acid constitutes 3–5% of the dry weight of cereal grains and legumes such as corn and soybean. The high concentration of phytates in animal feed and the inability of non-ruminant animals such as swine and poultry to digest phytates leads to phosphate contamination of soil and water bodies. The supplementation of animal feed with phytases results in increased bioavailability to animals and decreased environmental contamination. Therefore, phytases are of great commercial importance. Phytases with a range of properties are needed to address the specific digestive needs of different animals. Alkaline phytase (LlALP1 and LlALP2) which possess unique catalytic properties that have the potential to be useful as feed and food supplement has been identified in lily pollen. Substantial quantities of alkaline phytase are needed for animal feed studies. In this paper, we report the heterologous expression of LlALP2 from lily pollen in *Pichia pastoris*. The expression of recombinant LlALP2 (rLlALP2) was optimized by varying the cDNA coding for LlALP2, host strain and growth conditions. The catalytic properties of recombinant LlALP2 were investigated extensively (substrate specificity, pH- and temperature dependence, and the effect of Ca^{2+}, EDTA and inhibitors) and found to be very similar to that of the native LlALP2 indicating that rLlALP2 from *P. pastoris* can serve as a potential source for structural and animal feed studies.

Introduction

Phytases are a class of phosphatases that catalyze the sequential hydrolysis of phytic acid [*myo*-inositol hexakisphosphate (Fig. 1)] to less phosphorylated inositol phosphates, and in some cases, to inositol [1–6]. Inositol phosphates play multiple roles in biological processes including signal transduction and calcium regulation [7–11]. Phytic acid is the most abundant inositol phosphate in cells [12], thus phytases, the primary enzymes responsible for the hydrolysis of phytic acid, play important roles in the metabolism of inositol phosphates.

A number of phytases with varying structural and catalytic properties have been found in plants, yeast, bacteria, and animals [2,3]. The enzymes differ in pH optima (acidic and alkaline), catalytic mechanisms (histidine acid phosphatase-like phytase, purple acid phosphatase-like phytase, cysteine phosphatase-like phytase, and β-propeller phytase), substrate specificity, specificity of hydrolysis, metal ion requirements, susceptibility to inhibitors, and thermostability [1–4]. A novel alkaline phytase was detected in the pollen grains of *Lilium longiflorum* (LlALP) [13]. Cloning and sequence analysis identified two isoforms of alkaline phytase cDNA, *LlAlp1 and LlAlp2*, which are 1463 and 1533 bp long and encode proteins of 487 and 511 amino acids, respectively [14]. The calculated molar mass of LlALP1 and LlALP2 monomers are 53.8 and 56.2 kDa, respectively; the active enzyme exists as a homodimer. The deduced amino acid sequences revealed that the enzyme is a histidine phosphatase, it contains the signature heptapeptide of histidine phosphatases, –RHGXRXP– near the N-terminus [14].

In cereal grains and legumes phytate constitutes 3–5% of the dry weight of seeds [12]. During seed and pollen development, phytic acid is deposited in membrane-bound bodies as a salt of essential mineral ions such as potassium, calcium, magnesium, iron, and zinc and this then serves as a source of phosphate, inositol, and metal ions during the germination of seeds and pollen grains [12]. Although high concentrations of phytate are present in corn and soybean, the major components of animal feed, the phosphate, inositol, and essential metals in phytate complexes are unavailable to humans and monogastric animals, such as poultry, swine and fish, because they lack phytase [1–4,15,16]. Therefore, for optimum animal growth, supplementation of animal feed with phosphate and essential minerals, at additional cost, is necessary [16]. The excretion of undigested phytate from animals results in elevated levels of orga-

Abbreviations: IB, inclusion body; AOX1, alcohol oxidase; LiAc, lithium acetate.

* Corresponding author. Fax: +1 906 487 2061.
E-mail address: ppmurthy@mtu.edu (P.P.N. Murthy).

1046-5928/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2010.07.003

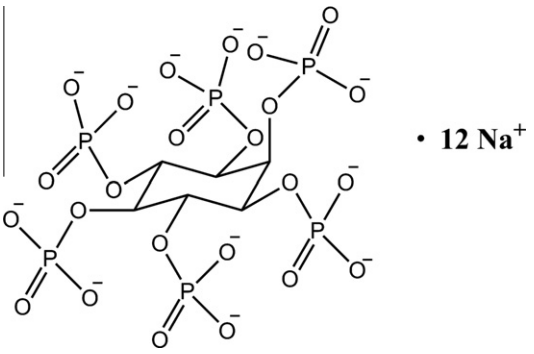

Fig. 1. Dodecasodium salt of phytic acid.

nophosphate in soil and water bodies downstream of agriculturally intensive areas [16]. To address these nutritional and environmental contamination issues, governments have passed legislation to supplement animal feed with phytases [4,15]. The efficacy of supplementing animal feed with phytase to increase the bioavailability of inositol and phosphates and decrease phosphorous discharge from animal farms has been demonstrated in several experiments [4,15,17]. The commercial importance of phytases in animal feed has resulted in the need for substantial quantities of an array of phytases with a range of catalytic and stability properties for use in different end applications [15]. Investigation of the biochemical properties of alkaline phytase from lily pollen indicates that the enzyme has unique catalytic properties and suggests that the enzyme has the potential to be useful as a feed and food supplement [18].

Our initial efforts at heterologous expression of rLlALP2 from lily pollen in *E. coli* resulted in low levels of expression and the accumulation of LlALP2 in inclusion bodies [14]. Although recombinant proteins in inclusion bodies (IB) are frequently inactive, alkaline phytases in IBs did exhibit catalytic activity [14]. Numerous efforts to solubilize alkaline phytase from IBs and refold them into the more active form were unsuccessful. Once denatured, rLlALP2 did not refold into the biologically active form under any of the numerous conditions [19] we tried (unpublished data); the fact that the enzyme is a homodimer with a total of 20 cysteine residues may have contributed to the refolding challenges we encountered.

The methylotropic yeast, *Pichia pastoris*, has been shown to be a good expression system for high-level production of intracellular and secreted foreign proteins [20–22]. Heterologous expression of proteins in *P. pastoris* has many advantages over bacterial expression hosts: *P. pastoris* is capable of posttranslational modifications such as folding, disulfide bond formation, and glycosylation; expression of foreign proteins is driven by the tightly controlled and highly inducible alcohol oxidase (*AOX1*) promoter; proteins can be targeted to the extracellular media serving as the first step in purification; it is relatively inexpensive compared to other eukaryotic expression systems; the ability to grow to high cell densities coupled with high expression rates results in high protein yields, expression levels can range from mg to many g per L of growth culture [21–24].

In this paper, we describe the heterologous expression of a plant alkaline phytase (lily) in *P. pastoris*. Our aim was to investigate the expression of the active enzyme in a soluble form in *P. pastoris*. As a first step, we decided to express the enzyme intracellularly so as to avoid the additional problems associated with the *Pichia* secretary pathway. In an effort to optimize the expression of LlALP2, the *LlAlp2* insert, host strain and growth conditions were varied. Catalytic properties of the recombinant enzyme (rLlALP2) expressed in *P. pastoris* were investigated and compared to the wild-type enzyme. To our knowledge, this is the first report of the heterologous expression of a phytase from a plant source.

Materials and methods

Strains, plasmids, and media

Escherichia coli TOP10F′, *P. pastoris* strains X-33 and KM71H, and expression vector pPICZA were purchased from Invitrogen (Carlsbad, CA). Primers were purchased from Integrated DNA Technologies (Coralville, IA) and GoTaq Flexi DNA polymerase was purchased from Promega (Madison, WI). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). Peptone, tryptone, yeast extract, glycerol, biotin, and methanol were purchased from Fisher (Pittsburgh, PA). Yeast nitrogen base was purchased from BD Biosciences (Sparks, MD). HisPur cobalt was purchased from Thermo Fisher (Rockford, IL).

Low salt LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) was used for propagation of *E. coli* (TOP10F′). Low salt LB medium plates (1.5% agar) containing kanamycin (25 μg/mL) (Research Products International Corp., Mt. Prospect, IL) were used to select for transformants of TOP10F′.

P. pastoris strains were grown in either BMGY (2% peptone, 1% yeast extract, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% glycerol) or BMMY medium (2% peptone, 1% yeast extract, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% methanol). YPD agar plates [1% yeast extract, 2% peptone, 2% dextrose, 2% agar, and various concentrations (100, 500, 1000 or 2000 μg/mL) of zeocin, as indicated] were used for selection of *P. pastoris* transformants.

Construction of expression vector

Alkaline phytase cDNA (*LlAlp2*) was amplified via PCR from a previously constructed plasmid [14]. Forward primers for *LlAlp2*-Ser (EcoRI restriction site in bold and the codons corresponding to Ser and Ala underlined) (5′-TAGCAT**GAATTC**AAAAATGTCTGC GTTCTCGCTTCACGC-3′) and *LlAlp2*-Ala (5′-TAGCAT**GAATTC** AAAAATGGCGTTCTCGCTTCACGC-3′) were used in conjunction with a reverse primer (NotI restriction site in bold, stop codon italicized) containing a stop codon (5′-AAAAGGAAAA**GCGGCCGC** *TCA*CAGTTCTGTCTTCTGGTTACCGGTG-3′) or without a stop codon (5′-AAAAGGAAA A**GCGGCCGC**CAGTTCTGTCTTCTGGTTACCGGTG-3′). Absence of the stop codon allowed for the incorporation of a *c-myc* epitope tag followed by a $(His)_6$ tag at the carboxyl terminus of the protein. PCR amplification (35 cycles of 94 °C for 30 s, 60 °C for 50 s and 72 °C for 2 min and a final elongation of 5 min at 72 °C) yielded a 1500 bp product coding for *LlAlp2*. The *LlAlp2* cDNA was purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified *LlAlp2* cDNA insert and pPICZA were separately digested, first with NotI for 18 h after which EcoRI was added and the mixture incubated for an additional 4 h, and then ligated with T4 DNA ligase (16 h at 14 °C) per manufacturer's instructions. Competent TOP10F′ were transformed with recombinant plasmids according to Sambrook [25] and positive transformants were selected on low salt LB agar plates containing 25 μg/mL zeocin.

The insertion of *LlAlp2* cDNA into pPICZA was confirmed by PCR. Plasmids were isolated using the Wizard® *Plus* DNA Purification System according to the manufacturer's instructions (Promega, Madison, WI). In brief, 5 mL of LB medium was inoculated with a single colony and grown overnight at 37 °C. Cells were collected by centrifugation (10 min at 1400*g*) and the plasmid extracted as suggested by the manufacturer. PCR amplification using plasmid DNA and *LlAlp2*-specific, full-length primers, used above, yielded a 1500 bp product corresponding to *LlAlp2* cDNA. A second PCR amplification using plasmid DNA and plasmid-specific primers that flank the insertion site (forward primer: 5′-GAC TGG TTC