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Expression and characterization of a recombinant unique acid phosphatase from kidney bean hypocotyl exhibiting chloroperoxidase activity in the yeast *pichia pastoris* ☆

Tohru Yoneyama [a], Maya Taira [a], Tomonori Suzuki [a], Masao Nakamura [b], Koichi Niwa [a], Toshihiro Watanabe [a], Tohru Ohyama [a,*]

[a] *Department of Food Science and Technology, Faculty of Bioindustry, Tokyo University of Agriculture, 196 Yasaka, Abashiri 099-2493, Japan*
[b] *Department of Chemistry, Asahikawa Medical College, Midorigaoka-Higashi 2-1-1-1, Asahikawa 078-8510, Japan*

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Abstract

We previously purified and characterized a novel acid phosphatase (KhACP) from kidney bean hypocotyls that exhibited vanadate-dependent chloroperoxidase (V-CPO) activity. In the present study, a functional recombinant KhACP (rKhACP) was successfully produced at a high expression level by the methylotrophic yeast *Pichia pastoris*. The KhACP cDNA excising signal peptide sequence was subcloned into the pPICZαA vector and then integrated into the genome of *P. pastoris* strain X-33 under control of the alcohol oxidase 1 promoter. The rKhACP protein, with a molecular mass of 60 kDa on SDS–PAGE, was secreted into the culture medium as a C-terminal His-tagged fusion protein. Purification was facile using only nickel affinity chromatography. The apparent molecular mass of the purified rKhACP was estimated to be around 110 kDa by analytical gel filtration. PAGE analysis showed that rKhACP was a glycosylated dimeric enzyme, consisting of two 60-kDa subunits linked non-covalently, which was similar to the dominant form of the natural enzyme isolated from plant material. Furthermore, the rKhACP exhibited V-CPO activity when ortho-vanadate (VO_4^{3-}) was added to the apo enzyme, and it showed broad substrate specificity and kinetic parameters comparable to the natural enzyme. This expression system produces sufficient protein to allow us to attempt to determine the three-dimensional crystal structure, which will shed light on its unique mechanism of converting KhACP to vanadate-dependent chloroperoxidase.

Keywords: Acid phosphatase; Chloroperoxidase; Vanadate; Kidney bean; Methylotrophic yeast *Pichia pastoris*

Acid phosphatase (ACP)[1] (EC 3.1.3.2) enzymes catalyze hydrolysis of phosphate monoesters, releasing inorganic phosphorus from phosphorylated substrates *in vitro* in the pH range 4–7. ACPs are ubiquitous in a wide variety of animals, plants and microorganisms, and they exhibit low substrate specificity [1,2]. Plant ACPs are present in various organs of germinating seeds and also in different cell compartments, suggesting that these enzymes are involved at various cellular metabolic levels. Plant ACPs are also induced under various environmental and developmental conditions, including salt or drought stress, seed germination, flowering and pathogen infection, making their cellular roles difficult to define [3–5]. However, the stimulation of phosphatase activities in response to phosphate starvation is well documented, and ACPs play a role in the utilization of phosphate compounds [1].

Purple ACPs are commonly found in a wide range of eukaryotic organisms and in some bacterial species. They

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* Corresponding author. Fax: +81 152 48 2940.
E-mail address: t-oyama@bioindustry.nodai.ac.jp (T. Ohyama).

[1] *Abbreviations used:* ACP, acid phosphatase; CPO, chloroperoxidase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PAS, periodic acid-Schiff; *p*-NPP, *p*-nitrophenyl phosphate; 2-ME, 2-mercaptoethanol; MCD, monochlorodimedon.

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belong to a family of non-specific ACPs containing a bimetal nucleus in their active center [6–8], that also includes phosphoprotein phosphatase and other types of phosphomonoesterase [9,10]. The characteristic purple color of the purified enzymes is the result of a tyrosine to Fe^{3+} charge transfer transition [11]. The purple colored ACP from the kidney bean is the best characterized plant enzyme, due to the determination of its three-dimensional structure. It is a homodimeric glycoprotein with a molecular mass of 110 kDa, containing a Fe^{3+}–Zn^{2+} metal site in each of two subunits that are connected by a disulfide bond [9,12].

Recently, we purified and characterized a novel colorless acid phosphatase (KhACP) from kidney bean (*Phaseolus vulgaris* cv. Ohfuku) hypocotyls, which was distinct from the kidney bean purple acid phosphatase [13]. When ortho-vanadate (VO_4^{3-}) was added to the apo-form of the enzyme, KhACP uniquely exhibited chloroperoxidase activity with concomitant loss of phosphatase activity. This is the first demonstration that KhACP is a vanadate-dependent chloroperoxidase (CPO) in plants, and it suggests that KhACP may play a role in modifying a wide variety of chlorinated compounds that are present in higher plants. KhACP is also a dimeric glycoprotein with a molecular mass of 96 kDa consisting of dominant 56 kDa and minor 45 kDa subunits that are connected by a non-covalent bond.

Unlike most purple ACPs that contain a binuclear Fe^{3+}–Zn^{2+} (Fe) center at their active sites [5], KhACP develops no purple color and shows CPO activity after the addition of vanadate. Additionally, Northern blot analysis demonstrated that expression of the *KhACP* gene was observed specifically in hypocotyls of the kidney bean, and that it coincided with elongation of the hypocotyl during germination, strongly suggesting that KhACP is clearly distinct from other ACPs that have been reported in plants. However, we have not obtained enough data to elucidate the physiological functions of KhACP, due to the limited yield of protein purified from the bean hypocotyl. Further characterization of the enzyme, such as determining its precise structure and physiological substrates, will require a large amount of KhACP.

We also isolated a gene encoding KhACP (GenBank Accession No. AB116719) from a kidney bean hypocotyls cDNA library [13]. The full-length *KhACP* gene is closely related to the soybean purple ACP-like gene *GmPAP3* [14] with 92% amino acid sequence identity, and to lupine ACP [15] with 87% sequence identity, but it has only 54% identity with the red kidney bean purple ACP gene [16]. Computer-assisted motif analysis and phylogenetic analysis suggest that KhACP belongs to a large purple ACP subfamily consisting of proteins with no disulfide bridge between their subunits. Nevertheless, KhACP is regarded as a novel ACP that differs from previously identified purple ACPs in plants.

The purpose of the present study is to establish an efficient system for expression of this unique KhACP, allowing us to produce milligram amounts of this protein. The methylotrophic yeast *P. pastoris*, which is one of the dominant expression systems in molecular biology due to its stable and high-level expression of heterologous proteins [17,18], can be easily grown to high cell density in defined minimal media. The yeast is also able to introduce eukaryotic post-translational modifications such as glycosylation. In the present work, we report our strategy for overexpression of this enzyme by *P. pastoris*. The cDNA encoding KhACP was successfully expressed in yeast cells as a His-tag fusion protein. The optimized purification procedure for obtaining milligram amounts of homogenous active recombinant enzyme is presented. We also confirm here that the expressed recombinant KhACP (rKhACP) is functionally equivalent to the natural enzyme.

Materials and methods

Yeast culture media

Pichia pastoris cells were cultured in YPD medium (1% yeast extract, 2% peptone, and 2% D-glucose) or BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol, 1% casamino acids, and 100 mM potassium phosphate, pH 6.0). YPDS plates (YPD medium plus 1 M sorbitol and 2% agar (w/v)) containing 100 μg/ml zeocin (Invitrogen, San Diego, CA) were used for selection of *Pichia* transformants. BMMY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 0.5% methanol, 1% casamino acids, and 100 mM potassium phosphate, pH 6.0) was used for protein induction.

Strains and vectors

Escherichia coli TOP10 cells and the plasmid vector pCR4-TOPO were used for cloning. For yeast transformation, the *P. pastoris* transfer vector pPICZαA containing the 5′ alcohol oxidase 1 (AOX1) promoter and the 3′AOX1 transcription termination sequences was used. pPICZαA also contains the dominant selectable marker zeocin, which is bifunctional in both *Pichia* and *E. coli*. *Pichia pastoris* host strain X-33 was used for protein expression experiments. These products were purchased from Invitrogen (Carlsbad, CA).

Construction of expression vector and P. pastoris transformation

The *KhACP* gene (AB116719) without a native signal peptide sequence (1275 bp) was amplified by PCR using kidney bean hypocotyls cDNA library as a template [13] with sense (5′-GAA TTC GGG ATC ACT AGC TCC TTC-3′) and antisense (5′-CTA GAT ACC AAT ACT GGT TAT GCA ATA C-3′) primers introducing an *Eco*RI site (sense) and *Xba*I site (antisense) (underlined, respectively) into the region immediately upstream of the first