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Rapid cloning, expression and purification of a novel high-activity alkaline phosphatase with detoxification of lipopolysaccharide

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ABSTRACT

Lipopolysaccharide (LPS) is a bacterial endotoxin leading to endotoxemia. Its virulence factor 'diphosphoryl lipid A' can be abolished by alkaline phosphatase (AP). A novel AP gene (without introns) was cloned from *Saccharomyces boulardii* ATCC MYA-796 with a GenBank accession number KF471017, and the recombinant AP (rAP) was expressed as a soluble protein in *Pichia pastoris* X-33 with a yield of 43.66 mg/l at the end of 120 h of induction in a shaker flask. After purification by affinity-column chromatography, the purity of rAP was over 90%. The optimal reaction conditions of rAP were pH 9.6, temperature at 60 °C and 2 mM Mg^{2+} in diethanolamine buffer, and EDTA was a potent inhibitor of rAP activity. The specific activity of rAP was 9912.01 U/mg under the optimal conditions. Furthermore, rAP showed a broad dephosphorylation activity to LPS over a broad pH range (pH 2–10) in vitro and peaked at pH 4 in Tris–HCl buffer. After LPS dephosphorylated by rAP was injected intraperitoneally into mice, the serum level of tumor necrosis factor (TNF)-α was significantly reduced compared to that of the LPS group ($p < 0.01$). These findings suggest that rAP has great potential to cure diseases caused by LPS.

1. Introduction

LPS is a major pathogenic constituent of the cell walls of Gram-negative bacteria. It is recognized by the innate immune system; triggers Toll-like receptor 4 (TLR4) signaling cascades through sequential binding to its co-receptors, such as LPS binding protein (LBP), CD14 and MD-2; and upregulates the expression of proinflammatory cytokines [1], resulting in endotoxemia and further causing systemic inflammatory syndrome, sepsis and multiple organ dysfunction syndrome [2–5]. However, Lu et al. reported that acyloxyacyl hydrolase deacylation cleaves acyl chains from the lipid A portion of LPS, preventing the prolonged systemic inflammatory reactions induced by LPS [6]. Additionally, if the diphosphoryl lipid A of LPS was converted into monophosphoryl lipid A by AP, dephosphorylated LPS also no longer activated TLR4 dependent inflammatory responses [7,8].

AP is a class of phosphate transferases that dephosphorylate LPS, ATP and nucleotides [9,10]. It is present in nearly all living microorganisms, and it consists of four different isoenzymes in mammals, including placental, placental-like, intestinal, and tissue-nonspecific isoenzymes. In the past, AP has been used as an indicator for early diagnosis of jaundice [11], rickets [12] and seminoma [13] etc. Much recent research has indicated that AP reduces the transmucosal passage of bacteria and increases intestinal tolerance to commensal bacteria [14–16]. AP also has the potential to cure diseases caused by LPS, such as inflammatory bowel disease [17], sepsis-induced acute kidney injury [18], neonatal necrotizing enterocolitis [19], chronic colitis [20] and secondary peritonitis [21], because it attenuates the toxicity of LPS by interrupting the LPS-mediated inflammation signaling pathway. Therefore, obtaining an effective and safe AP is an urgent need.

S. boulardii is used as a probiotic and biotherapeutic agent in clinical applications for prophylaxis and treatment of diarrheal diseases in both children and adults [22]. In addition to maltase [23] and spermine [24], *S. boulardii* also secretes a 63 kDa AP, which is stronger at dephosphorylating LPS of *Escherichia coli* 055B5 than either bovine or rat intestinal AP [25]. However, the production of AP from *S. boulardii* was very low, and the purification process was complex and costly [25]. By contrast, heterologous systems such as yeasts have proven to be successful for expression of eukaryotic proteins.

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The *P. pastoris* expression system has been successfully used to express many heterologous proteins [26–29], and it has the advantages of posttranslational modification, promoting formation of disulfide bonds, processing target proteins into native conformations and secretion of the recombinant proteins into the fermentation broth, which is beneficial for purification [29,30]. Meanwhile, the expression levels of target recombination protein can be improved significantly by high-cell-density fermentation in the *P. pastoris* expression system. Nevertheless, some APs have been expressed in *E. coli* expression systems as inclusion bodies [31,32]. It is common knowledge that the renaturation process of inclusion bodies is complex and inefficient.

In this study, a novel AP gene was cloned from *S. boulardii* and then heterologously expressed in *P. pastoris* X-33. Enzymatic properties of rAP, including effects of Mg^{2+}, EDTA, temperature and pH, were evaluated, as were the thermal stability and specific activity of rAP in optimum conditions. Additionally, the ability of rAP to reduce inflammation caused by LPS was investigated.

2. Materials and methods

2.1. Materials and mice feeding procedure

S. boulardii ATCC MYA-796 was purchased from American Type Culture Collection (ATCC). *E. coli* DH5α (Invitrogen, Beijing, China) was used as the host strain for vector construction, and the pPICZαA plasmid (Invitrogen, Beijing, China) and *P. pastoris* X-33 strain (Invitrogen, Beijing, China) were used for cloning and expression, respectively. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (NEB, Beijing, China). The kits for DNA purification and plasmid or yeast genome extraction were purchased from Tiangen (Beijing, China). LPS from *E. coli* 055B5 was purchased from Sigma. The TNF-α ELISA kit was purchased from Shanghai Elisa Biotech Co., Ltd. Other chemical reagents were analytical grade. SPF grade of ICR male mice were purchased from Vital River (Beijing, China). All mouse experiments were carried out in accordance with EU Directive 2010/63/EU for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm), and mice were reared in a ventilated animal caging system with controlled lighting (12 h light, 12 h dark) [33].

2.2. Cloning of a novel AP gene from S. boulardii rapidly

To obtain the DNA sequence of AP from *S. boulardii*, degenerate primers (S: 5′-TCTTGGTGACTGAYGGNATGGG-3′; A: 5′-ACCACCAGTYTCRTGRTC-3′) were designed with an online tool (http://blocks.fhcrc.org/blocks/make_blocks.html), based on two conservative regions that were identified by aligning 19 *Saccharomyces* AP protein sequences with ClusterW1.8 software. *S. boulardii* genomic DNA was isolated as the template for PCR using degenerate primers, and DNA fragments from *S. boulardii* were confirmed by sequencing and analyzed by Blast (http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Another pair of primers (F: 5′-CATTTACATACCAGCATTACGGGAC-3′; R: 5′-AAGAGGGAGAGTTAGATAGGAT-3′) was designed according to the sequence which had the most similarity to the DNA fragment from *S. boulardii*, and the *S. boulardii* AP gene was then amplified (using primers F and R) and sequenced.

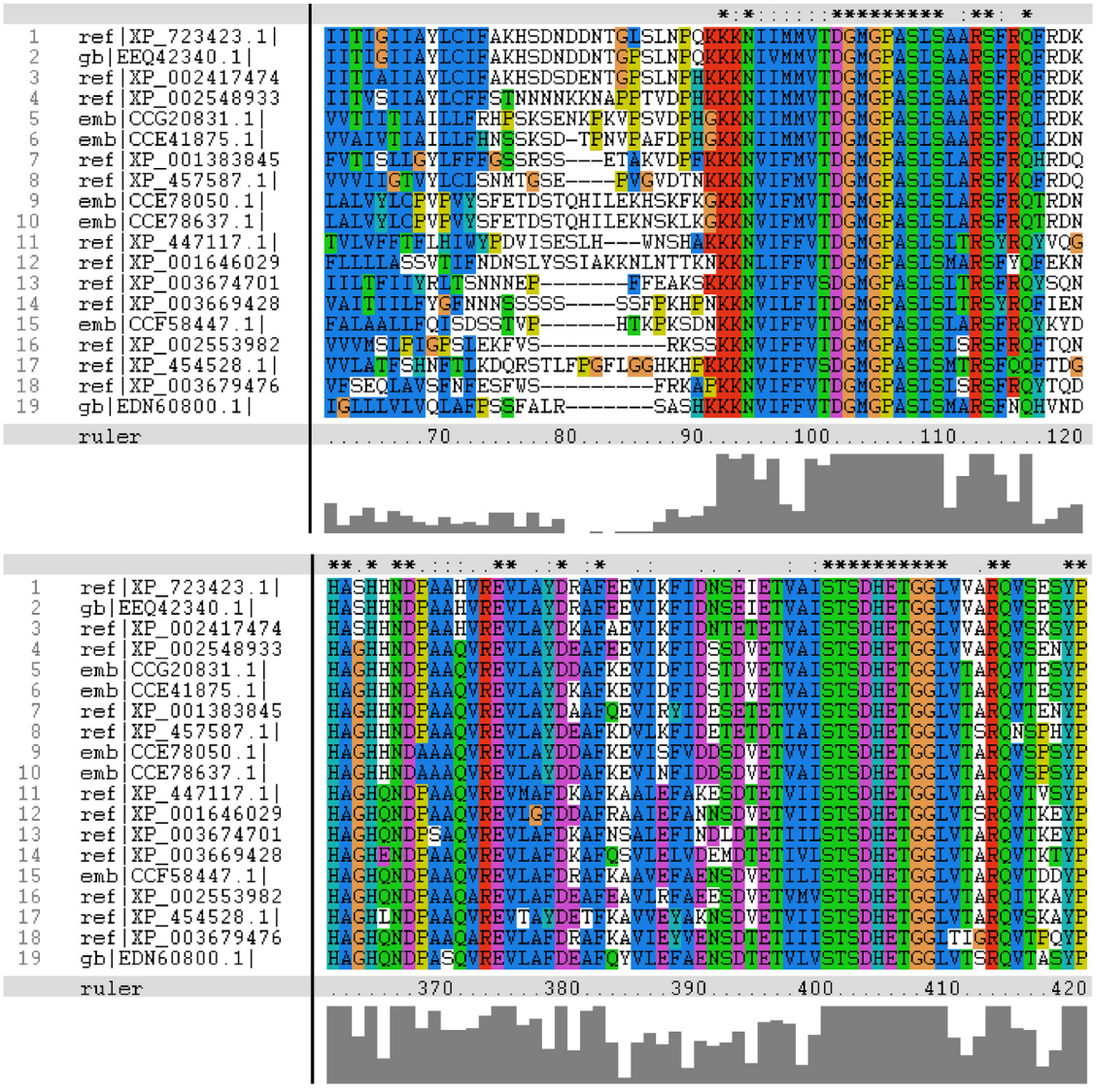

Fig. 1. Sequence alignment of *Saccharomyces* AP. Two conserved regions of *Saccharomyces* APs were analyzed with ClusterW1.8 software. * represents the conserved positions.