Recombinant expression and purification of an Oxysterol Binding Protein from *Aspergillus oryzae* 3.042

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Abstract. A full-length cDNA encoding a candidate Oxysterol-binding protein(OSBP) from *Aspergillus oryzae* (AoOSBP) was cloned and expressed in Escherichia coli as a maltose-binding protein (MBP) fusion protein. The MBP-AoOSBP protein from the importantly industrial fungus *A. oryzae* was purified by amylose resin and chromatography column. SDS-PAGE showed that MBP-AoOSBP has an estimated molecular weight of 182 kDa. OSBP and its homologues (ORPs) own the affinity for oxysterols, cholesterol and glycerophospholipids. According to the superiority of *A. oryzae* in the fermented foods and also in food-grade productions pharmaceutical enzyme manufacture, it is meaningful to identify the biochemical properties of OSBP in *A. oryzae*.

1 Introduction

Aspergillus oryzae, as a kind of GRAS (generally recognized as safe) strain, has been widely used for the production of certain traditional fermented Asian foods and alcoholic beverages [1] .This safe strain is also wildly being used for production of various industrial enzymes including amylases, proteases, lipases and phytases etc. [2]. The genome of *A. oryzae* has been sequenced in 2005 [3], which has facilitated research efforts to understand its basic biology and develop better industrial strains of this significant fungus [4] .

The study of *A. oryzae* genome database reveals the presence of a presumptive Oxysterol-binding protein (AoOSBP) coding gene which has a predicted open reading frame (ORF) 3792 bp capable of encoding a polypeptide of 1263 amino acids. Oxysterol-binding protein (OSBP), a cytoplasmic protein, is widely distributed in eukaryotes. OSBP and its homologues (ORPs) have been confirmed owning affinity for oxysterols, cholesterol and glycerophospholipids, due to their properties of the lipid-binding/transfer [5-9]. In yeast *Saccharomyces cerevisiae* are gene/protein family consists of seven members (designated Osh)[10], while in mammals including humans there are 12 ORP genes/proteins [11-13]. Some researchers studied the vertebrate model to illustrante the function of conservative OSBP-related proteins, known as lipid binding/transfer proteins, between zebrafish and

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human [14].

Until now, no investigation concerning OSBP from filamentous fungi has been documented. According to the superiority of *A. oryzae* in the fermented foods and also in food-grade productions pharmaceutical enzyme manufacture, it is meaningful to identify the biochemical properties of OSBP in *A. oryzae* as well as its expression level in the process of growth. In this study, the AoOSBP gene was cloned, recombinantly expressed in *E. coli* cells and the MBP-AoOSBP fusion protein were purified by mylose resin and chromatography column. purified. It is beneficial to the further research with the combined effect of phytosterol and its application in the fermentation process.

2 Materials and methods

2.1 Strains, plasmids and biochemical reagents

Escherichia coli JM109 and *Aspergillus oryzae* 3.042 were stored in Tianjin University of Science & Technology. *E. coli* OverExpress C43 (DE3) was bought from Shanghai Microgene Biotech Co.,Ltd. Vector PMAL-c4x (Invitrogen, USA) was used for fusion expression of AoOSBP protein from *A. oryzae*. Amylose resin was purchased from NEB (USA) and the chromatography column was from QIAGEN (Germany). Reagents and chemicals used for DNA manipulation and RNA extraction were purchased from Takara (Dalian, China). All the other reagents were of the analytical grade.

2.2 Total RNA extraction and reverse transcription

In order to acquire fungal mycelia for total RNA isolation, an approximately 1×10^7 spore suspension was inoculated into 50 mL Yeast Extract Peptone Dextrose Medium in a 250 mL flask and allowed to grow at 30 ºC for 24 h with shaking and then fungal mycelia were harvested and washed with deionized water. To isolate total RNA, mycelia were ground in liquid nitrogen in an ice-cold mortar and 0.1 g of the resultant mycelium powder was used for total RNA extraction with TRIzol® reagent (Promega, USA) according to the manufacturer's instructions. The quality and integrity of RNA was determined by 1% agarose gel electrophoresis. Then 3μg of total RNA was subjected to reverse transcription using random heptamer primers with the manufacturer's instructions. Next, the resultant cDNA was then used for PCR amplification of AoOSBP cDNA.

2.3 Construction of expression vector

To express AoOSBP as a maltose-binding protein (MBP) fusion, the pMAL-c4x vector (New England Biolabs) was used. Briefly, the full open reading frame (ORF) of AoOSBP was amplified from cDNA synthesized from mycelium total RNA using primer pairs with a *Sal*I restriction site incorporated at 5' end (5'-gtcgac TGGCCTGGTCGCGATAGTGATTCG-3') and a *Pst*I restriction site at the 3' end (3'-ctgcagCCTGGAGCAGGTACGCTCCAACCA-5'). High-fidelity Probest DNA polymerase (TaKaRa) was used to minimize potential mutations introduced during amplification. The thermocycler conditions were 95 ºC for 2 min; 30 cycles of 95 ºC -1 min, 55 ºC -1 min, 72 ºC -1 min; and afinal extension at 72 ºC for 10 min. The PCR product was first cloned into T vector and verified by sequencing, followed by digestion with *Sal*I and *Pst*I to release the OSBP gene fragment. pMAL-c4x vector (New England Biolabs) was linearized with *Sal*I and *Pst*I, and the OSBP insert was ligated into it following digestion with the same enzymes. The ligation product was transformed into *E. coli* JM109 cells (Invitrogen, USA) and transformants were selected on LB plates supplemented with 100 μg/mL ampicillin. The resultant recombinant expression plasmid, designated pMAL-AoOSBP, was confirmed by digestion of appropriate restriction enzymes and subsequent sequencing.

2.4 Expression of recombinant OSBP in *E. coli*

The expression plasmid pMAL-AoOSBP was transformed into *E. coli* OverExpress C43 (DE3) cells for AoOSBP expression. Briefly, a single colony of *E. coli* transformant was inoculated into 50 ml LB broth containing 100 μg/mL ampicillin in a 250 mL flask. After overnight cultures at 37 ºC with shaking at 220 rpm, 4 mL cells were inoculated into 400 mL fresh LB medium containing 100 μg/mL ampicillin in a 1 L flask and grown at 37 ºC with shaking. When cell OD_{600} reached 0.6-0.8 putting the flask on the ice 15 min, IPTG was added to a final concentration of 0.5 mM and the speed was slowed down to 160 rpm. The cells were harvested after a further 20 h incubation at 13 ºC.

2.5 Purification of AoOSBP

Crude protein preparations and all further work with OSBP were carried out at 4ºC. After cultivation, cells were collected by centrifugation at 5,000 × g for 15 min and pellets resuspended in 20 mL binding buffer (20 mM Tris-HCl, 200 mM NaCl, 1mM EDTA, pH 7.4). Cells were lysed by sonication(15 min cycle of 3 s on, 3 s off, at 45% amplitude) on the ice and cell debris removed by centrifugation (40,000 × g, 4 ºC, 30 min). After centrifugation, the supernatant was filtered and mixed gently for 1 h at 4 ºC with 2 mL of amylose resin (NEB) that was equilibrated in binding buffer. Then the mixture was flowed through the chromatography column, and repeated this step for two or three times. The non-adsorbed protein fraction was eluted from the column with binding buffer and the remaining protein eluted with a step gradient of maltose . Target protein fractions were pooled, concentrated using an Amicon Ultra-4 unit (MWCO 30 kDa), and stored at -80 ºC for subsequent analysis.

3 Results and Discussion

3.1 Cloning of the AoOSBP gene from A. oryzae

A large eukaryotic gene family with homology to OSBP has been revealed by analysis of genomic and cDNA databases [15-17]. OSBP is characterized for two domains, the pleckstrin homology (PH) domain at the N-terminus, and the ligand-binding (LB) domain at the C-terminus. The PH domain, which is found in many membrane-binding proteins, mediates membrane association of the OSBP through phosphoinositide interaction [18,19], and The C-terminal domain that in some cases has been shown to bind oxysterols, cholesterol and ergosterol [20,21].

For expression of AoOSBP protein in *E. coli* cells, the full-length 3792 bp cDNA was amplified by RT-PCR from *A. oryzae* mycelium total RNA (Figure 1). The sequence of the amplified AoOSBP cDNA fragment was confirmed in the GENEWIZ. Then the amino acid sequence of AoOSBP was carried on the comparison in the NCBI BLAST, and the result is an exact match with *Aspergillus oryzae* 3.042, as shown in Figure 2. The gene of AoOSBP was connected to the expression vector pMAL-c4x to express the MBP-AoOSBP fusion protein in *E. coli* cells. The Figure 3 showed that pMAL-AoOSBP expression vector was constructed successfully.

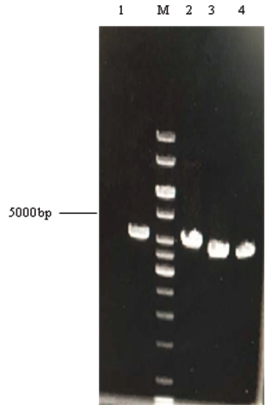

Figure 1. The agarose gel of the genome and cDNA of AoOSBP. M: KB Ladder; Lane1 and Lane 2: The genomic size of AoOSBP (~ 4000bp); Lane 3 and Lane 4: The cDNA size of AoOSBP (~3800bp).

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics Distance tree of results Multiple alignment

Description	Max score	Total score	Query cover	E value	Ident	Accession
oxysterol-binding protein [Aspergillus oryzae 3.042]	2606	2606	100%	0.0	100%	EIT77350.1
oxysterol binding protein (Osh1) [Aspergillus oryzae RIB40]	2603	2603	100%	0.0	99%	XP_001825518.1
oxysterol binding protein [Aspergillus flavus AF70]	2601	2601	100%	0.0	99%	KOC17205.1
hypothetical protein ANOM_001793 [Aspergillus nomius NRRL 13137]	2373	2373	99%	0.0	94%	XP_015411210.1
oxysterol binding protein (Osh1) [Aspergillus fumigatus Z5]	2001	2001	100%	0.0	78%	KMK55954.1
oxysterol-binding protein homolog C2F12.05c [Aspergillus udagawae]	1981	1981	100%	0.0	78%	GAO85745.1
oxysterol binding protein [Aspergillus kawachii IFO 4308]	1975	1975	100%	0.0	78%	GAA91102.1
unnamed protein product [Aspergillus niger]	1956	1956	100%	0.0	78%	CAK40942.1
oxysterol binding protein [Aspergillus niger]	1954	1954	99%	0.0	78%	GAQ46371.1

Figure 2. The results of Comparing in the NCBI BLAST shows the gene of AoASBP is extracted from the *Aspergillus oryzae* 3.042.

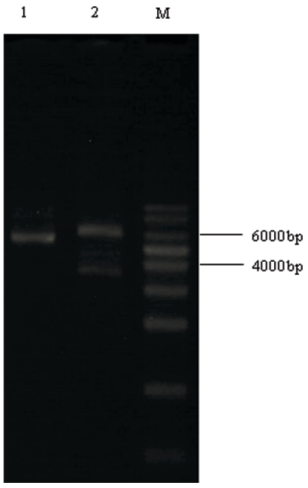

Figure 3. The agarose gel of expression vector pMAL-AoOSBP. M: KB Ladder; Lane 1: The plasmid of pMAL-AoOSBP; Lane 2:The pMAL-AoOSBP digested by SalI and PstI (The size of vector is 6645bp and the gene is 3792bp).

3.2 Heterologous expression and purification of AoOSBP

To determine the induction conditions for optimal AoOSBP expression in OverExpress C43 (DE3) cells, various IPTG concentrations and incubation times were examined. It was found that under the condition of 0.5 mM IPTG and incubation at 13ºC for 20 h was suitable for AoOSBP expression (data not shown). And under the same condition , added inducers and without it the fusion protein expression of the target protein have obvious differences(Figure 4 A). To purify MBP-AoOSBP protein, the crude protein preparation was combined with amylose resin at 4 ºC and the mixed was throughed chromatography column, then eluted with the washing buffer. The purified MBP-AoOSBP was analyzed by SDS-PAGE (Figure 4 B). Based on the SDS-PAGE, the gene of OSBP from *A. oryzae* was successfully expressed in *E. Coli.*

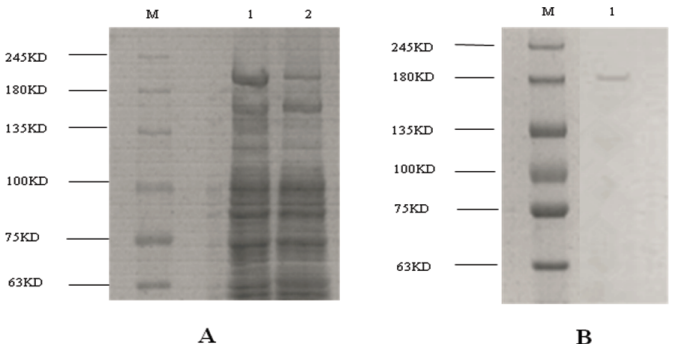

Figure 4. 8% SDS-PAGE analysis of the MBP-AoOSBP fusion protein. (A) Lane M: protein molecular weight marker. Lane 1: The expression of the MBP-AoOSBP fusion protein after adding inducers; Lane 2: The broken supernatant that without inducers. (B) lane M is the protein marker; lane 2 shows purification of AoOSBP by the amylose resin and chromatography column.

4 Summary

Strains of *A. oryzae* are used for the production of a number of popular traditional Asian fermented foods, in particular for large-scale manufacture of soy sauce whose unique flavors are favored by consumers. It is well known that *A. oryzae* plays a significant role in the taste and smell of its fermented final product and lipid metabolism contributes to the flavor formation during fermentation. OSBP is a lipid-binding protein that has been implicated in the regulation of various cellular processes, including nonvesicular cholesterol transport, signaling, lipid metabolism, and vesicular trafficking. In this study, we successfully obtained target gene of AoOSBP through reverse transcription-polymerase chain reaction. The sequence of the amplified AoOSBP gene were confirmed by sequencing. Then the AoOSBP gene were connected to the expression vector pMAL-c4x to express the MBP-AoOSBP fusion protein in *E. coli* cells. The MBP-AoOSBP fusion protein were purified by amylose resin and chromatography column.

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