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Full Length Article

Characterization of an Acyl-CoA Binding Protein from *Zygosaccharomyces rouxii*

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Abstract

To understand the structure and function of acyl-CoA binding protein (ACBP) from *Zygosaccharomyces rouxii* (*Zr*ACBP), the acyl-CoA binding protein of *Saccharomyces cerevisiae* (*Sc*ACBP) was chosen for comparison. The acyl-CoA binding protein (ACBP) revealing 261 bp open reading frame and encoding 87 amino acids with molecular mass of 10 kDa. The amino acid sequence of *Zr*ACBP exhibited high sequence identity (80%) with that of *Sc*ACBP. Besides, the structure of *Zr*ACBP was molded based on structure of *Sc*ACBP, which also contained four-helix bundles that act as the peptide backbone. But, there have a great differences in the hydrophobicity and the subcellular localization. In order to verify the difference function of ACBPs in two yeasts *in vitro*, the recombinant maltose-binding protein (MBP)-*Sc*ACBP and MBP-*Zr*ACBP protein was purified and binded with fatty acyl-CoA esters by Microscale thermophoresis. The results indicated that the K_D values of *Sc*ACBP binding with fatty acyl-CoA esters were lower one order than that *Zr*ACBP, which suggested the *Zr*ACBP binding with fatty acyl-CoA esters is only a fraction of its function and is involved more in other metabolism of lipid. © 2019 Friends Science Publishers

Keywords: Acyl-CoA-binding protein; *Zygosaccharomyces rouxii*; Fatty Acyl-CoA esters; Microscale thermophoresis

Introduction

Acyl-CoA-binding protein (ACBP) is a highly conserved protein, which has high specific affinity with saturated and unsaturated medium and long-chain fatty acid acyl-CoA esters. ACBP also has a four-helix bundle that binds with fatty acyl-CoA esters and transports to subcellular structures where it is required. For example, ACBP is able to transport fatty acyl-CoA esters and donate into mitochondria *β*-oxidation, glycerol biosynthesis and phosholipid synthesis (Rasmussen *et al.*, 1994; Knudsen *et al.*, 2003). ACBP also protect long-chain acyl-CoA esters from hydrolyzing by cellular acyl-CoA hydrolases. In short, the protein plays the role of receptors for medium and long-chain acyl-CoA esters, and it is also a weak donor of acyl-CoA (Kragelund *et al.*, 1999). Research into the homologous gene in yeast has shown that ACBP is necessary for fatty acid chain elongation, sphingolipid synthesis, protein sorting and vesicle transport (Gaigg *et al.*, 2001). Recent studies have reported that ACBP could resistant the adverse environment, such as the drought (Du *et al.*, 2013).

The salt-tolerant yeast *Z. rouxii* plays a critical role in the flavour formation during brine fermentation (Golden *et al.*, 1994; Van *et al.*, 2001). It can synthesize flavour components: Ethanol, higher alcohols, One 4-hydroxyfuranone derivative, 4-hydroxy-2 (or 5) -ethyl-5(or 2) -methyl-3 (2H) -furanone (HEMF) and other esters that impart the specific flavour to soy sauce and to some extent determine the quality of the sauce (Yokotsuka, 1986; Sasaki *et al.*, 1991). To date, little research has been conducted to the relationship between the genes of *Z. rouxii* related to fatty acid metabolism and the resulting aroma components. Such ACBP is an important intermediate fatty acid metabolic, that it is interesting to investigate the function of *Zr*ACBP. In this study, the structure of *Zr*ACBP was predicted on the basis of *Sc*ACBP structure and the function of ACBPs from two yeasts were verified by Microscale thermophoresis (MST). It's our purpose to compare the differences of structure and function of two heterologous ACBPs. This was constructed the foundation for us to continue to research the relationship between aroma components and fatty acid metabolism in *Z. rouxii*.

Materials and Methods

Strains and Plasmids

Strains: *E. coli* DH5α and *Rosetta* DE3 (Invitrogen, USA) were used as host strains for cloned and expressed the recombinant protein. The *S. cerevisiae* (CICC 1273, China) and the *Z. rouxii* (Strain Collection Center of Jiangxi Science and Technology Normal University, China) were provided to extract total RNA.

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Plasmids: The PMAL-c4x (New England Biolabs, USA) plasmid was used to express the MBP-ACBP fusion protein. Fatty acyl-CoA esters (Avanti, USA) acted as the ligand to bind with recombinant protein. All reagents used for gene amplification and recombinant plasmid construction were purchased from Takara (Takara, China). All experiments were carried out at least three times.

Constructing the Recombinant Plasmid

Constructing the recombinant plasmid: For RNA isolation RNAkit (Omega, USA), for cDNA preparation PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, China) were used. cDNA was diluted 1:5 with RNase-free H_2O before PCR amplification. The ACBP gene amplification of two kinds of yeast were carried out using gene specific forward and reverse primers (Supplementary Table 1), the PCR fragments were confirmed by DNA sequencing. And then, the fragments were cloned into a pMAL-c4x vector to express the MBP fusion proteins. Furthermore, the recombined vectors were confirmed by digestion of appropriate restriction enzymes.

Expression and Purification of Recombinant Protein

Expression of recombinant protein: Recombined vector was transformed into *E. coli* Rosetta (DE3) cells for MBP-ACBP expression. Briefly, a single colony of *E. coli* transformant was inoculated into 10 mL LB (1% Bacto Tryptone, 0.5% yeast extract, 1% NaCl pH 7) supplemented with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. After overnight cultivation, 5 mL cells were inoculated into 400 mL fresh LB at 37°C with shaking 200 rpm/min. When cell OD_{610} reached 0.4–0.6, isopropylthio- β -D-galactoside (IPTG) was added to 0.5 m*M* for expressing of the recombinant protein. The cells were harvested by centrifugation (8,000*g, 4ºC, 5 min) after a further 20 h incubation at 16ºC.

Purification of recombinant protein: The whole experiment was carried out at 4ºC. At first, the harvested cells were resuspended in binding buffer (20 m*M* Tris – HCl, 200 m*M* NaCl, 1 m*M* EDTA, pH 7.4). The cells were disrupted by sonication and the supernatant was collected by centrifugation (13,000*g, 4ºC, 30 min). And then, the supernatant was loaded onto a previously equilibrated amylose resin chromatography column and the column was then washed with 2 volumes of binding buffer, the bound proteins were eluted with 2 mL of elution buffer (20 m*M* Tris – HCl, 200 m*M* NaCl, 1 m*M* EDTA, pH 7.4, 5 m*M* maltose) (Duongly and Gabelli, 2015; Hao *et al.*, 2016). The purified protein was quantified by SDS-PAGE and BCA assay.

Binding Assay

MST is an all-optical approach to characterize the properties of biomolecules, which based on thermophoresis, the directed motion of molecules in temperature gradients. A Monolith NT. 115 system was used to measure the bind constants (K_D values) of nine acyl-CoA esters binding with two recombinant proteins. In order to eliminate interference factors of labeled protein, the buffer of purified protein was exchanged into labeling buffer, and then, the purified proteins were labeled using the Monolith NT Protein Labeling Kit RED (NanoTemper Technologies) following manufactures instructions.

A range of concentrations of the required ligand (ranging from 450 uM to13.7 nM) was respectively incubated with 100 nM labeled protein 5 min in bingding buffer. The samples were loaded into the standard treated capillaries and microthermophoresis carried out using 40% LED power and 20% MST power. The binding curves were fitted and the K_D values were calculated by the Affinity Analysis (x86) which was known to give fast and accurate results (Seidel *et al.*, 2013).

Results

Cloning of *acbp* Gene from *S. cerevisiae* and *Z.rouxii*

The CDS of *Sc*ACBP and *Zr*ACBP was obtained from cDNA of *S. cerevisiae* and *Z. rouxii* by RT-PCR and confirmed by DNA sequencing. Phylogenetic tree was constructed (PAM250) using MEGA 5 software (Tamura *et al*., 2014) with the Maximum Likelihood tree and bootstrap analysis was performed for 1000 replicates. The results revealed that the homology of both proteins were much higher than that of other Fungus (*Aspergillus oryzae* and *Tortispora caseinolytica*) (Fig. 1A). The multiple sequence alignment of ACBP protein was performed by CLUSTALW method, the results show that they had high similarity and conservatism (Fig. 1B).

Protein sequence of two ACBPs were analyzed for physicochemical characteristics (http://www.expasy.org/tools), subcellular localization (http://psort.hgc.jp/). The results shown that their molecular weight like most ACBP is about 10KDa. However, it is significant difference that the subcellular of two kinds of yeast's ACBP (Table 1). The *Sc*ACBP is mainly located in the cytoplasm like most ACBPs, while the *Zr*ACBP is mainly located in the mitochondria. The hydrophilicity of ACBPs were analyzed with the PSORT Server (http://psort.hgc.jp/form2.htm1), which shown that both of them have obvious hydrophobic regions, but the hydrophobicity of *Zr*ACBP lower than that of *Sc*ACBP (Fig. 2). In addition, the two proteins displayed no transmembrane segments and signal peptide cleavage site (Supplementary Fig. 1 and 2), which might be cytosolic in nature like other species'ACBP. Especially, the *S. cerevisiae* was cultured with starvation medium, the ACBP could secrete extra-cellular with the unconventional way which related to the yeast autophagy (Cuff and Barton, 2000).

Table 1: Analysis of two kinds of proteins' physico-chemical properties and subcellular localizatio

Species	Encoding amino acids	Molecular weight(Da)	Isoelectric point(Ip)	Subcellular localization(percentage)				
				Nuclear	Mitochondrial	Cytoskeleton	Cytoplasmic	Others
*Sc*ACBP	87	10059.6	4.56	17.40%	13.00%	4.30%	65.20%	0.10%
*Zr*ACBP	87	10336.9	4.75	47.80%	47.80%	4.30%	0.10%	

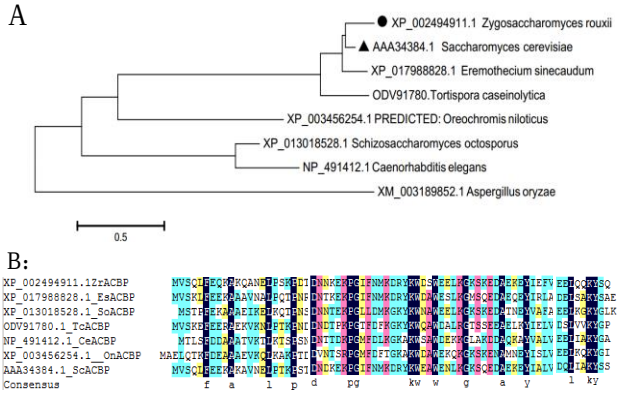


Fig. 1: Construction of phylogenetic tree and analysis of homology
Fig. 1A: The ACBPs of different fungi species were selected to construct the phylogenetic tree, numbers before the species abbreviations are the accession numbers in GenBank
Fig. 1B: Multiple alignment of the conserved region in the ACBP domain between *Zr*ACBP and other representative eukaryotic ACBPs. Amino acids shared between *Zr*ACBP and other sequences are shaded, while conserved residues among all listed sequences are labelled in the bottom row

As a conserved protein family, ACBP contains four typical 4-helice bundles which are the domain of ACBP protein. The tertiary structure of *Z. rouxii* ACBP was developed with the Swiss-MODEL (http://swissmodel.expasy.org/) and JPred prediction server (Duran *et al.*, 2010). The *S. cerevisiae* ACBP (NCBI Accession number, AAA34384.1) was used as a template, which was that most homologous to *Zr*ACBP (PDB Entry 1ST7, showing 80% sequence identity with *Zr*ACBP, Fig. 3A and B) and the reliability analysis were given in Supplementary Fig. 3. The structure of *Zr*ACBP is similar to common ACBP, 4 helices bundles also act as the domain which arranged in an up-down-down-up fold, where helices A1 and A2, A3 and A4 are connected with a short turn, A2 and A3 are connected by long random coil and located "front" of the whole protein, the helix A4 is the longest in whole helixs and bends slightly toward the helix A1- helix A4 interface.

Expression and Purification of MBP Fusion Proteins

For expression of ACBPs as an MBP fusion protein, *Escherichia coli* Rosetta (DE3) was transformed with plasmids containing the appropriate construct. MBP-*Sc*ACBP, MBP-*Zr*ACBP and MBP protein were purified by amylose resin chromatography column.

The purified MBP-*Sc*ACBP, MBP-*Zr*ACBP and MBP protein were analysed by SDS-PAGE with a separation gel concentration of 12%. The result shown that the purified

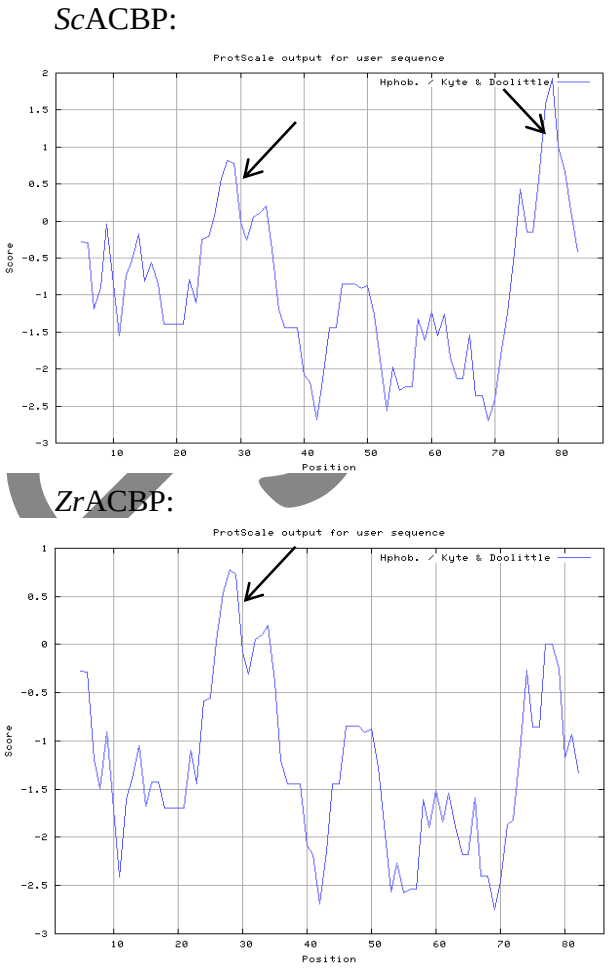


Fig. 2: Analysis of hydrophobic and hydrophilic of two yeasts' ACBP
The arrow points to the hydrophobic region is obvious. The *Sc*ACBP contains two distinct hydrophobic regions, but the *Zr*ACBP only has one

fusion protein was estimated to be 50 kDa and the MBP-tag was about 40 kDa (Fig. 4). This implied that the molecular weight of ACBP was about 10 kDa, which is consistent with the prediction by DNAMAN software.

Identification of Biochemical Function of Recombinant Protein by MST

The Monolith NT. 115 system was used to measure the ability of nine acyl-CoA esters binding with recombinant proteins. The purified MBP tag was acted as the control and did not significantly bind with fatty acyl-CoA esters; further details were shown in Supplementary Fig. 4.

Table 2: The K_D values of recombinant protein binding with fatty acyl-CoAs

Illuminant	Iigand	*Kd* value (nM)	
		Labeled fluorescent protein (MBP-ScACBP)	Labeled fluorescent protein (MBP-ZrACBP)
NT-647	Hexanoyl(C6:0)-CoA	4155± 766	13731 ±3788
	Octanoyl(C8:0)-CoA	312 ± 94	9204±1545
	Decanoyl(C10:0)-CoA	258 ± 78	4012±600
	Dodecanoyl(C12:0)-CoA	175± 47	1184±169
	Myristoyl(C14:0)-CoA	132± 58	739±116
	Palmitoyl(C16:0)-CoA	311 ± 107	2193±639
	Stearoyl(C18:0)-CoA	154 ± 86	3421±1000
	Eicosanoyl(C20:0)-CoA	128 ±65	1456±645
	Behenoyl(C22:0)-CoA	123±73	2025±501

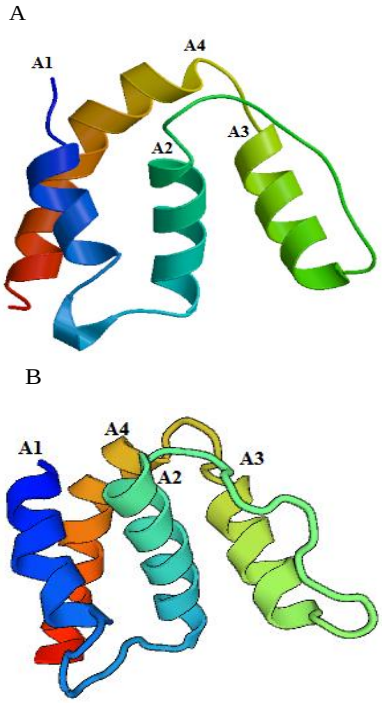


Fig. 3: Establishing the 3D model of *Zr*ACBP
Fig. 3A: The 3D structure of ScACBP was obtained from Protein Data Bank entry 1ST7. B: The 3D model of *Zr*ACBP was established, the four-helix bundles also formed the main frame, helix A4 is the longest fragment compared other helices. However, A1 rendered discontinuous helices

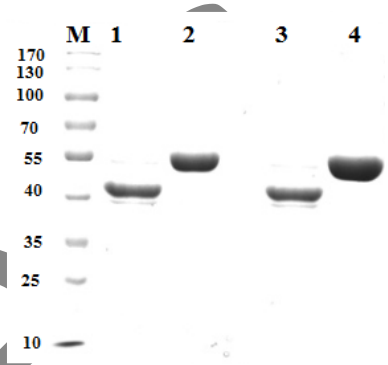


Fig. 4: Staining 12 % SDS-PAGE gel shows the purification protein on an amylose resin chromatography column
M: protein markers; Lane 1: MBP tag control; Lane 2: MBP-*Sc*ACBP; Lane 3: MBP

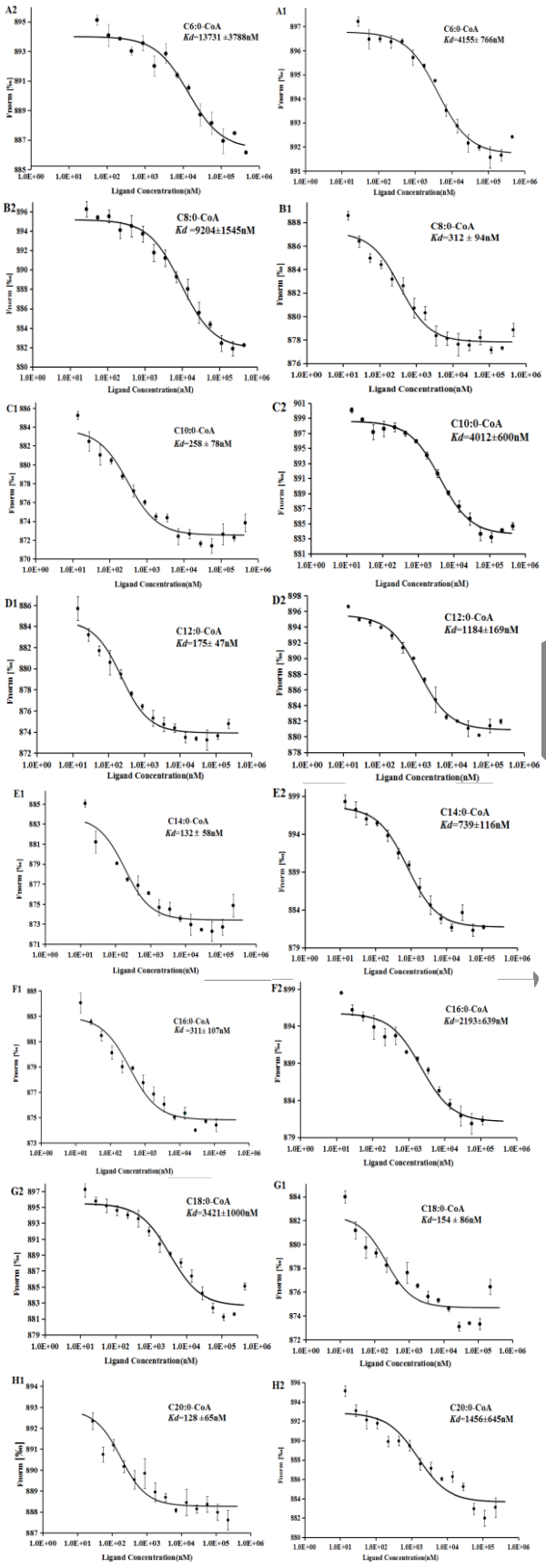
The binding curves and K_D values of MBP-*Zr*ACBP and MBP-*Sc*ACBP were shown in Fig. 5 and Table 2. As we know, the smaller the values of K_D, the stronger ability of acyl-CoA ester binding with ACBP. Surprisingly, the K_Ds of *Zr*ACBP binding with ligands were higher than that *Sc*ACBP, meaning that the *Zr*ACBP binding with acyl-CoA esters were looser than that *Sc*ACBP. Compared with the lower *Kd* values (nM level) for most ACBPs to long-chain acyl CoA reported, the K_Ds of MBP-*Zr*ACBP reached uM level, which suggested that the function of the *Zr*ACBP binding with acyl-CoA ester is only a fraction of its function.

The *Sc*ACBP binding with fatty acyl-CoA esters has been reported. Researchers had found that C16:0-; C18:0-; C20:0- CoA esters had affinity for *Sc*ACBP (Gaigg *et al.*, 2001), as well as our binding assay, from hexanoyl (C6:0) - CoA to dodecanoyl(C12:0) - CoA, with the increase carbon chain of acyl-CoA ester, the ability of binding recombinant protein was enhanced. Besides, long-chain fatty acyl CoA esters and *Sc*ACBP have tighten binding ability, but there is a slight difference from C14:0 to C22:0- CoA esters.

In brief, the ACBP portion of the MBP-ACBP fusion protein is able to specific recognition ligands. The ACBP clearly shows a binding preference for medium and long chain fatty acyl-CoA esters. There are differences in function of ACBP with different species, which may be associated with other functions of the species.

Discussion

In order to compare the function of *Zr*ACBP with *Sc*ACBP, the K_D values of the two proteins with nine acyl-CoA esters were detected by MST assay. It is worth noting that the K_D values of *Zr*ACBP were higher an order than that *Sc*ACBP. Thus, a question arises that whether *Zr*ACBP participated more in other fatty acid metabolism compared with binding acyl-CoA esters. In high salt fermentation conditions, *Z. rouxii* can survive in the salt concentration up to 18% (Sluis *et al.*, 2001), which induced a series of cascade reactions, for example: HOG - MAPK pathway (Edmunds and Mahadevan, 2004) and relies on the Ca^{2}+/CaM calcineurin (CaN) pathway (Dina *et al.*, 1997). These pathways promote the synthesis of glycerol and change in the composition of cell membranes, to accommodate extracellular high salt concentration.



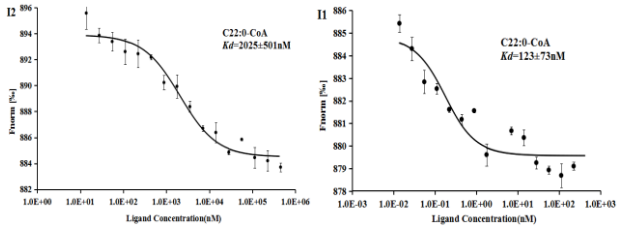


Fig. 5: The purified proteins binds with nine of Acyl-CoA esters
tag control; Lane 4: MBP-*Zr*ACBP
The figures of A1-I1 were shown the binding curves and k_D values of MBP-*Sc*ACBP; The figures of A2-I2 were shown the MBP-*Zr*ACBP

Reports have been verified the ACBP was associated with the synthesis of glycerol, which mainly acted as a signaling molecule to regulate the Ca^{2+} ion channels and the gene expression (Neess *et al.*, 2015). In addition, ACBP also influences the synthesis of sphingolipids (Gault *et al.*, 2010) and phospholipids (Gaigg *et al.*, 2001). These two substances are the important components of the cell membrane. In a certain extent, it suggested that ACBP was involved in the stress response of yeast in high osmotic pressure. Thus, *Z. rouxii* can also make many acidic, high-sugar and canned foods get deteriorated, causing losses to the food industry. What's more, the hydrophilicity acted as a decisive factor of protein stability (Kyte and Doolittle, 1982). The hydrophilicity of *Zr*ACBP is higher than that of *Sc*ACBP, which may affect the binding site of *Zr*ACBP with fatty acyl-CoAs.

Therefore, the identification of *Zr*ACBP in vitro will serve as a significative point to better understand the relationship between lipid metabolism, contents of flavor compounds and others *Zr*ACBP functions.

As shown in Fig. 5 and Table 2, the K_D values of *Zr*ACBP binding with ligands were higher than *Sc*ACBP, so the ability of *Zr*ACBP to bind with long-chain fatty acyl-CoA is weaker than that of *Sc*ACBP. It means that the long-chain fatty acids produced by *Z. rouxii* are more easily released. Also, lipids account for a large proportion in the flavor substances of Soy sauce. These released fatty acids combine with alcohol to produce esters, which explains why the volatile flavors after fermentation of *Z. rouxii* are much richer than those after *S. cerevisiae* fermentation.

Exploring the effect of *Zr*ACBP on the flavor substances in Soy sauce fermentation has important research significance. Next, we will over-express *Zr*ACBP in yeast expression system, or knock-out *Zr*ACBP gene for changing the formation of fatty acid-derived flavors and aroma compounds, which will lead to the remoulding of *Z. rouxii* with desirable fermentation traits in terms of product flavor profiles.

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