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Influence of Tags on the Binding Affinity of Acyl-CoA Binding Protein

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Abstract: Tagged fusion proteins are frequently employed for protein purification methods, but their effects on protein function and binding affinity are rarely studied. Here we expressed recombinant protein Acyl-CoA Binding Protein (ACBP) cloned from the full-length cDNA of *Aspergillus oryzae* and *Saccharomyces cerevisiae*. ACBP was expressed in *Escherichia coli* fused to a Maltose-Binding Protein (MBP) and Histidine-tag fusion. Recombinant ACBP was purified using affinity chromatography columns and high protein purity was achieved. Microscale Thermophoresis (MST) binding assays showed that recombinant AoAcbp1 had a greater affinity for Palmitoyl-CoA (Kd = 35 nM) and Stearoyl-CoA (Kd = 23 nM) whilst recombinant ScAcbp had a greater affinity for Myristoyl-CoA (Kd = 31 nM) and Palmitoyl-CoA (Kd = 51 nM). In addition, MBP tagged ACBP had comparable binding affinities to His-tagged ACBP. Taken together, these data highlight that the size of the tagged fusion protein does not influence protein ACBP binding affinity.

Keywords: Acyl-CoA Binding Protein, Tagged Fusion Protein, Affinity Chromatography, Microscale Thermophoresis, Affinity Analysis

Introduction

Aspergillus oryzae and *Saccharomyces cerevisiae* are frequently used in industrial biotechnology (Liu *et al*., 2014) for fatty acid biosynthesis and metabolism, particularly in the fermented food industry (Piras, 2014). *A. oryzae* is used in the fermentation of rice-based food, soy and sake (Knuf and Nielsen, 2012), due to its high levels of protein secretion and ability to perform post-translational modifications (Meyer *et al*., 2011). In addition, *S. cerevisiae*, commonly known as the fermented yeast of bread and beer, is widely used in food production (Yvert *et al*., 2003). As the most studied genetic model species, the whole genome sequences of *A. oryzae* and *S. cerevisiae* are widely available (Machida *et al*., 2005; Wei *et al*., 2007).

The biosynthesis and degradation of microbial fatty acids is important during fermentation, *A. oryzae* and yeast always consumed and transformed lipids of soy during its fermentation (Fukushima *et al*., 1991). Fatty acid oxidation is mediated by fatty acyl-CoA and acyl-CoA Binding Protein (ACBP) (Wei *et al*., 2007). ACBP is widely expressed in eukaryotic organisms and pathogenic bacteria (Vock *et al*., 2006) where is participates in the intracellular transport of acyl-CoAs (Knudsen *et al*., 2000). Previous studies have characterized the functions of ACBP in *A. oryzae* and *S. cerevisiae*. In *A. oryzae*. ACBP (AoAcbp1) encodes a polypeptide of 365 amino acids that mediates fatty acid metabolism through its interaction with acyl coenzyme A, contributing to the flavor of soy sauce through the metabolism of esters (Bouyakdan *et al*., 2015). Recently, Kwon *et al*. (2017) identified another ACBP (AoAcbp2) from *A. oryzae*, was likely essential for growth. In Hao *et al*. (2016) indicated that AoAcbp1 has a higher affinity for Palmitoyl-CoA and Stearoyl-CoA, revealed that AoAcbp1 is a long-chain ACBP in *A. oryzae*. In *S. cerevisiae*, ACBP (ScAcbp) is a highly conserved 10 kDa protein that is required for vacuole function and ceramide synthesis (Faergeman *et al*., 2004), ScAcbp activity reduces the levels of hydroxy-C26:0 fatty acids, influences sphingolipid synthesis and regulates the expression of genes involved in fatty acid desaturation (Gaigg *et al*., 2001). Amino acid sequence homologies between AoAcbp2 with AoAcbp1 and ScAcbp are 12.6% and 26.4%, respectively. AoAcbp2, is widely conserved in fungi and AoAcbp1, is only seen in filamentous fungi, but not in yeasts (Kwon *et al*., 2017).

For further study of ACBP, prokaryotic expression and purify is critical. It is convenient for us to study the function, structure and other properties of proteins. Proteins with high purity, simple culture processes, rapid growth and low media costs, are frequently used for protein expression studies. During protein purification, tagged fusion proteins are used for convenience. Maltose-Binding Protein (MBP) is a periplasmic protein of *E. coli* involved in chemotaxis and the uptake of maltose sugars (Reichenwallner *et al*., 2013). MBP was inserted downstream of the *malE* gene, resulting in the expression of an MBP fusion (Duplay *et al*., 1984). Amylose resin can be used as an affinity matrix to purity the MBP tagged protein (Sambrook and Russell, 2006). His-tags contain 6-tandem histidine residues that do not influence the solubility or biological function of target proteins (Vijayalakshmi, 2008). N-terminal His-tagged proteins can be purified using one-step Immobilized Metal-chelate Affinity Chromatography (IMAC) (Li *et al*., 2017;Wang *et al*., 2017). In past studies, when MBP was used as fusion protein, whether it would affect the function of ACBP, it was uncertainty. Thus, we selected two common tagged fusion proteins for the purification.

In this study, MBP-ACBP and His-tagged ACBP were produced to high purity for the assessment of their binding affinities using Microscale Thermophoresis (MST) technology. Our findings were in agreement with results obtained using ITC and NMR (Pagano *et al*., 2008). The Monolith NTTM Protein Labeling Kit permits NT-647-NHS labeling of small amounts of protein (2-20 mM). Respective K_d values were calculated using the mess action equation via Nano Temper software from duplicate reads of triplicate experiments (Wienken *et al*., 2010). MST was shown to efficiently determine such weak interactions between small fragments and proteins in our system. We reproducibly performed experimental binding measurements using minimal amounts of protein and fragments (Gudim *et al*., 2017).

Materials and Methods

Strains, Plasmids and Biochemical Reagent

Escherichia coli DH5α, *E. coli Rosetta* (DE3), *A. oryzae* 3.042 and *S. cerevisiae* S288c were grown in the Jiangxi Province Key Laboratory of Bioprocess Engineering. Vector Pet-28a(+) and PMAL-c4x (Invitrogen, USA) were used to construct recombinant plasmids. Experiment reagents for RNA extraction and molecular biology procedures were purchased from Takara (Dalian, China). Nickel affinity columns and Isopropyl-β-D-1-Thiogalactopyranoside (IPTG) were purchased from QIAGEN (Beijing, China). Yeast extract, tryptone, NaCl and amylose resin were purchased from Sangon Biotech (Shanghai, China). Ni-Agarose Resin was purchased from CWBIO (Beijing, China). Phenylmethanesulfonyl Fluorode (PMSF) was purchased from Beyotime Biotechnology (Shanghai, China). Each fatty acyl-CoA was purchased from Avanti Polar Lipids, Inc. (Alabama, USA). The Monolith NT^{TM} Protein Labeling Kit was purchased from NanoTemper (Germany).

Cloned ACBP and Construction of the Expression Vector

AoAcbp1 was cloned from *A. oryzae* 3.042 and ScACBP was cloned from *S. cerevisiae* S288c. pMAL-c4x and Pet-28a vector were used to express recombinant AoAcbp1 and ScAcbp fusion proteins,. Vector Pet-28a(+) has a His-tag in its N-terminus and pMAL-c4x has an MBP in its N-terminus. Pet-28a(+) was used for the expression of the His-tagged AoAcbp1 and ScAcbp fusions. pMAL-c4x was used to produce MBP fusions of AoAcbp1 and ScAcbp.

We found that ACBP from *A. oryzae* and *S. cerevisiae* encoded proteins of 365 and 87 amino acids in length, respectively. Both proteins contained an ACBP domain, as predicted by the Pfam program (http://pfam.xfam.org). This domain accounted for only a small portion of AoAcbp1. According to predictions from the Pfam program, we expressed a protein that contained domain fragments. This was initially cloned by PCR from *A. oryzae* cDNA using the following primers: AoAcbp1-domain for 5 ' GAATTCATGTCGGACTCTGTGAGGTATGCGTCGC AGACGCC 3 ' , Ao Acbp1-domain Rev 5 ' AAGCTTCTAGGGGCATTTATGATACCTTGG 3 ' . Underlined sequences were attached for Multisite Gateway cloning. The fragment was ligated to pMAL-c4x to select single clones, confirmed by diagnostic restriction analysis and PCR.

Recombinant Protein Expression

E. coli is used for recombinant protein expression as it does not inherently secrete proteins into the extracellular environment (Sommer *et al*., 2009). We constructed a series of expression vectors to facilitate extracellular protein production and purification.

pMAL-AoAcbp1, pMAL-ScAcbp, Pet-AoAcbp1, Pet-ScAcbp and pMAL-AoAcbp1-domain were transformed into *E. coli* Rosetta (DE3) cells for recombinant expression and the plate count method used to identify transformed cells. Monoclonal colonies were grown in 50 mL LB containing antibiotics in 250 mL conical flasks. (pMAL-AoAcbp1, pMAL-ScAcbp and the pMAL-AoAcbp1-domain, media were supplemented 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. For Pet-AoAcbp1, Pet-ScAcbp added 100 μg/mL kanamycin and 34 μg/mL chloramphenicol were added for selection). Following overnight culture, 2 mL of cells were added into 200 mL LB in a 500 mL conical flask, which was shaken at 25°C. When the OD_{610}

reached 0.4-0.6, 0.5 mM IPTG was added and cells were collected after 16 h culture at 18°C.

Purification of Fusion-Tagged Proteins

For purification, cultured cells centrifuged at 12000 rpm for 20 min and resuspended in binding buffer on ice. Cells were sonicated for 10 min, centrifuged at 12000 rpm for 20 min and supernatants were collected. Recombinant proteins were purified using affinity chromatography columns.

pMAL-AoAcbp1, pMAL-ScAcbp, pMAL-AoAcbp1-domain possessed tagged MBP proteins with affinity for adsorption on amylose resin chromatography columns. MBP fusions remained bound in binding buffer (20 Mm Tris-HCl, 200 Mm NaCl, 1 Mm EDTA, pH 7.4) and were eluted in buffer containing 5 mM maltose.

Pet-AoAcbp1, Pet-ScAcbp have His-fusions with affinity for adsorption on Ni-NTA columns. His-tagged proteins remained bound to the column in the presence of washing buffer and were eluted by buffer containing 250 mM imidazole. Purified proteins were confirmed by SDS-PAGE and Coomassie blue staining.

Microscale Thermophoresis Binding Assays

The use of the Monolith NT^{TM} Protein Labeling Kit, provides a convenient method of labeling purified AoAcbp1 and ScAcbp. Column A (for buffer exchange) permits protein purification in a buffer that lacks contain primary amines or imidazole. Proteins were mixed with NT-647-NHS dye 1:1 ratio (200 µL final volume) and incubated for 30 min at room temperature in the dark. Column B (gravity flow column for purification) was used to remove unbound dye and eluted fractions were assessed for fluorescent intensity suing the Monolith device. For this assays, 16 serial dilutions of the sample were produced and diluted 1:1 with fluorescently labeled protein. Sample were mixed for 5 min at room temperature and loaded into the NanoTemper glass capillaries to initiate MST analysis in a NanoTemper monolith NT.115 (California USA).

As a control, we expressed Pmal-c4x without ACBP and identically to pMAL-ScAcbp, to obtain MBP, verified whether MBP has affinity with each fatty-acids.

Results and Discussion

Cloning of the ACBP from *A. oryzae* and *S. cerevisiae*

AoAcbp1 from *A. oryzae* 3.042 was 77% identical to *A. fumigatus*, *A. clavatus*, *A. nomius* and 99% identical to *A. flavus* (Yao *et al*., 2016). AoAcbp1 (GenBank accession number Ao3042_01296) was cloned and expressed as a MBP-AoAcbp1 fusion (purified by a step gradient of maltose) and a His-AoAcbp1 fusion (purified with a step gradient of imidazole). GFP-tagged AoAcbp1 localizs to punctate structures in the cytoplasm and is transported to vacuoles via the autophagy machinery (Kawaguchi *et al*., 2016). AoAcbp2 displays diffuse cytoplasmic staining (Kwon *et al*., 2017). ScAcbp cDNA (GenBank accession number YGR037C) from *S. cerevisiae* S288c, a highly conserved sequence (Gaigg *et al*., 2001), was cloned and expressed as an MBP-ScAcbp fusion and His-ScAcbp fusion and purified as described for AoAcbp1. Multiple alignment of the AoAcbp1 domain from several species revealed its importance to acyl-CoA binding activity, particularly Tyr and Lys residues which are evolutionarily conserved (Hao *et al*., 2016). Fragments containing the AoAcbp1-domain were confirmed by sequencing analysis (Sangon Corporation).

Prokaryotic Expression and Protein Purification

The expression vector was transformed into *E. coli* Rosetta (DE3) for protein expression. Bacteria were induced under varying temperatures and IPTG concentrations to ensure optimal purification conditions, confirmed by SDS-PAGE. Induction with 0.5 mM IPTG at 18°C produced the highest levels of recombinant proteins expression. To purify MBP tagged proteins, crude samples were loaded into amylose resin chromatography columns which were eluted with washing buffer. A Ni-agarose resin column was used for and His-tagged fusion proteins. Purified fusions were analyzed by SDS-PAGE.

MBP-AoAcbp1 had a molecular weight of ~ 82 kDa (Fig. 1, lane.1), MBP-ScACBP = 52 kDa (Fig. 1, lane.2), His-AoAcbp1 = 40 kDa (Fig. 1, lane.3), His-ScACBP = 10 kDa (Fig. 1, lane.4), MBP-AoAcbp1-Domain = 53 kDa (Fig. 1, lane.5), MBP = 42 kDa (Fig. 1, lane.6), All fusion proteins were of the predicted molecular weight.

Microscale Thermophoresis Binding Assays

Gravity flow and spin columns were used to purify the labeled proteins. MST analysis revealed that MBP-AoAcbp1 and His-AoAcbp1 had a binding preference for Stearoyl-CoA and Eicosanoyl-CoA (Table 1) (K_d values and response curves are shown in Fig. S1 and S2) and the linear relativity shown that K_d values (MBP-AoAcbp1 and His-AoAcbp1 with each fatty acyl-CoA) had no significant difference and had good linear correlation (Fig. 2A). MBP-ScAcbp and His-ScAcbp had a binding preference for Myristoyl-CoA and Palmitoyl-CoA (Table 2) (K_d values and response curves are shown in Fig. S3 and Fig. S4) and the linear relativity shown that K_d values (MBP-ScAcbp and His–ScAcbp with each fatty acyl-CoA) had no significant difference and had good linear correlation (Fig. 2B). In addition, from the K_d values (C4-C20), the presence of MBP or the His-tag did not influence the acyl-CoA binding affinity. The MBP-AoAcbp1-domain bound strongly to fatty acyl-CoA (C12:0-C18:0) (Fig. 3). As a control, MBP had no binding affinity with fatty acyl-CoA (C12:0-C18:0) (Fig. 4).

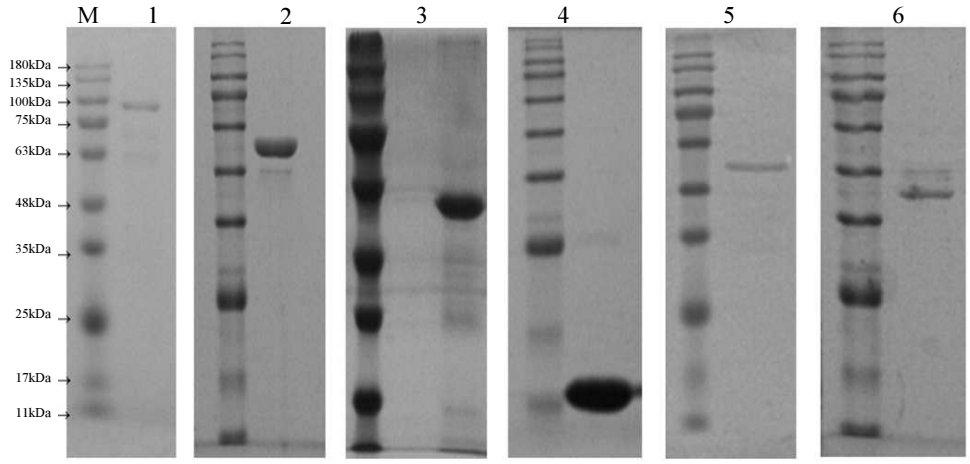

Fig. 1: Coomassie staining of purified recombinant proteins; M protein marker, 1 MBP-AoAcbp1, 2 His-AoAcbp1, 3 MBP-ScAcbp, 4 His-ScAcbp, 5 MBP-AoAcbp1-domain, 6 MBP

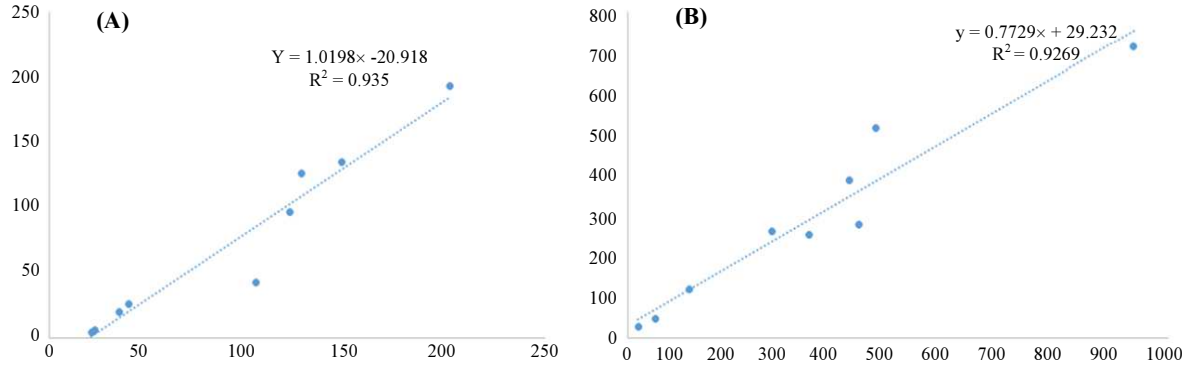

Fig. 2: Linear correlation curve (A) the linear relativity of K_d values (MBP-AoAcbp1 and His–AoAcbp1 with each fatty acyl-CoA) (B) the linear relativity of K_d values (MBP-ScAcbp and His–ScAcbp)

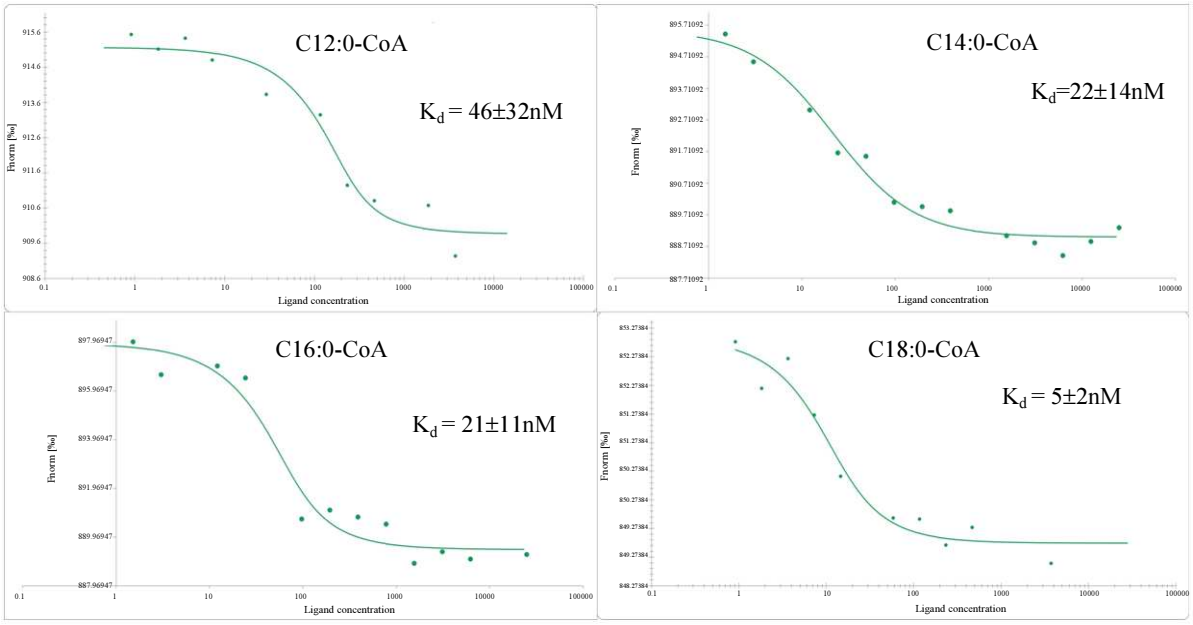

Fig. 3: Concentration response curve for MBP–AoAcbp1-domain ligand binding assessed by MST

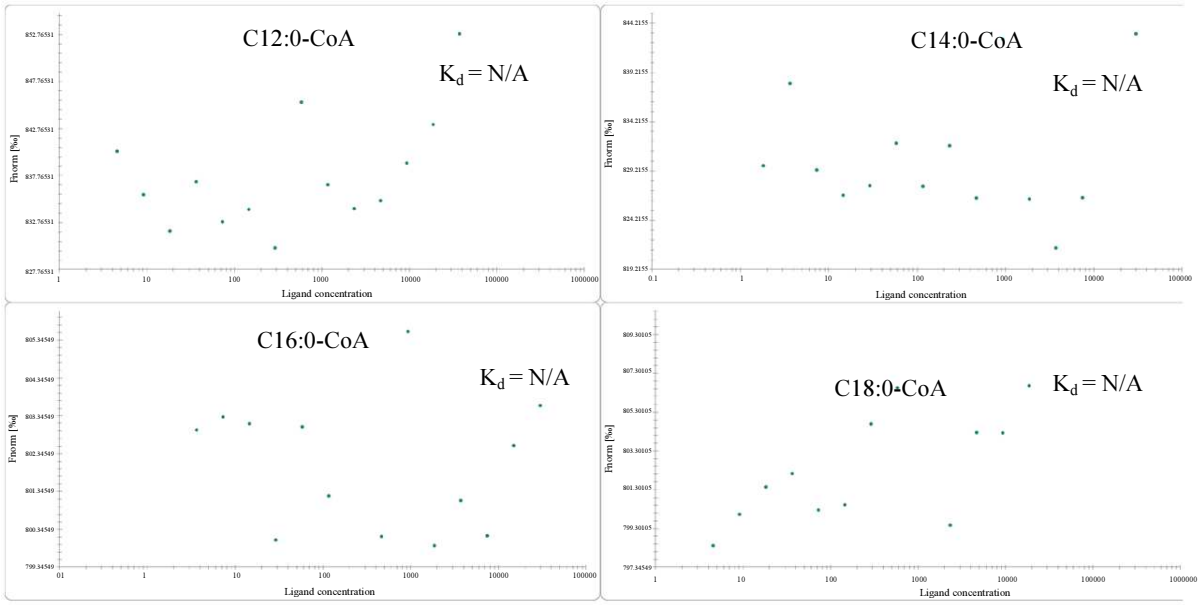

Fig. 4: Concentration response curve for MBP ligand binding assessed by MST

Table 1: MST assessment of K_d values revealing the binding affinity of MBP-AoAcbp1 and His–AoAcbp1 with each fatty acyl-CoA

Numbers	Carbon numbers	Species	K_d value of MBP-AoACBP (nM)	K_d value of His-AoACBP (nM)
1	C4:0	Butyryl-CoA	202±114	193±130
2	C6:0	Hexanoyl-CoA	147±84	135±123
3	C8:0	Octanoyl-CoA	127±99	126±244
4	C10:0	Decanoyl-CoA	121±62	96±71
5	C12:0	Dodecanoyl-CoA	104±29	42±19
6	C14:0	Myristoyl-CoA	40±17	26±22
7	C16:0	Palmitoyl-CoA	35±14	20±14
8	C18:0	Stearoyl-CoA	23±12	6±2
9	C20:0	Eicosanoyl-CoA	21±11	4±3

Table 2: MST assessment of K_d values revealing the binding affinity of MBP-ScAcbp and His-ScAcbp with each fatty acyl-CoA

Numbers	Carbon numbers	Species	K_d value of MBP-ScACBP (nM)	K_d value of His-ScACBP (nM)
1	C4:0	Butyryl-CoA	936±500	716±186
2	C6:0	Hexanoyl-CoA	460±175	514±268
3	C8:0	Octanoyl-CoA	410±190	387±216
4	C10:0	Decanoyl-CoA	267±144	262±199
5	C12:0	Dodecanoyl-CoA	114±68	119±72
6	C14:0	Myristoyl-CoA	20±17	26±24
7	C16:0	Palmitoyl-CoA	51±20	45±30
8	C18:0	Stearoyl-CoA	335±187	251±88
9	C20:0	Eicosanoyl-CoA	428±206	278±129

The MST data revealed that AoAcbp1 has a high specificity and affinity for long-chain acyl-CoA esters. Thus AoAcbp1 is a long chain ACBP. Furthermore, no binding was detected for each fatty acyl-CoA to the MBP tag (Hao *et al*., 2016), demonstrating that binding is AoAcbp1 specific. AoAcbp1 and ScAcbp had a preference for binding relatively long chain acyl-CoAs, that conform to the fatty acid composition of *A. oryzae* 3.042 and S. cerevisiae S288c.

In summary, we provide insight into the binding affinity and specificity of AoAcbp1 and ScAcbp for fatty acyl-CoA. Comparable affinity was observed between MBP and His-tags of ACBP from *A. oryzae* and *S. cerevisiae*. In addition, no non-specific binding of the inserted tags was observed. To date, protein crystallization typically employs His-tagged fusions for protein purification. As no influence of the tags was observed, MBP-tagged fusions are also applicable for crystallography studies and do not influence.

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Author's Contributions

Jizhong Han: Has contributed in whole experiment, data analysis, paper writing and publishment.

Yunlong Sun: Has assisted in the experiment and coordinated the data-analysis.

Haoran Li, Mengmeng Liu and Yu Chen: Have contributed to the acquisition of data and modification of the manuscript.

Bin Zeng: Has conceived and designed the experiments and contributed to get support for this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and there are no ethical issues involved.

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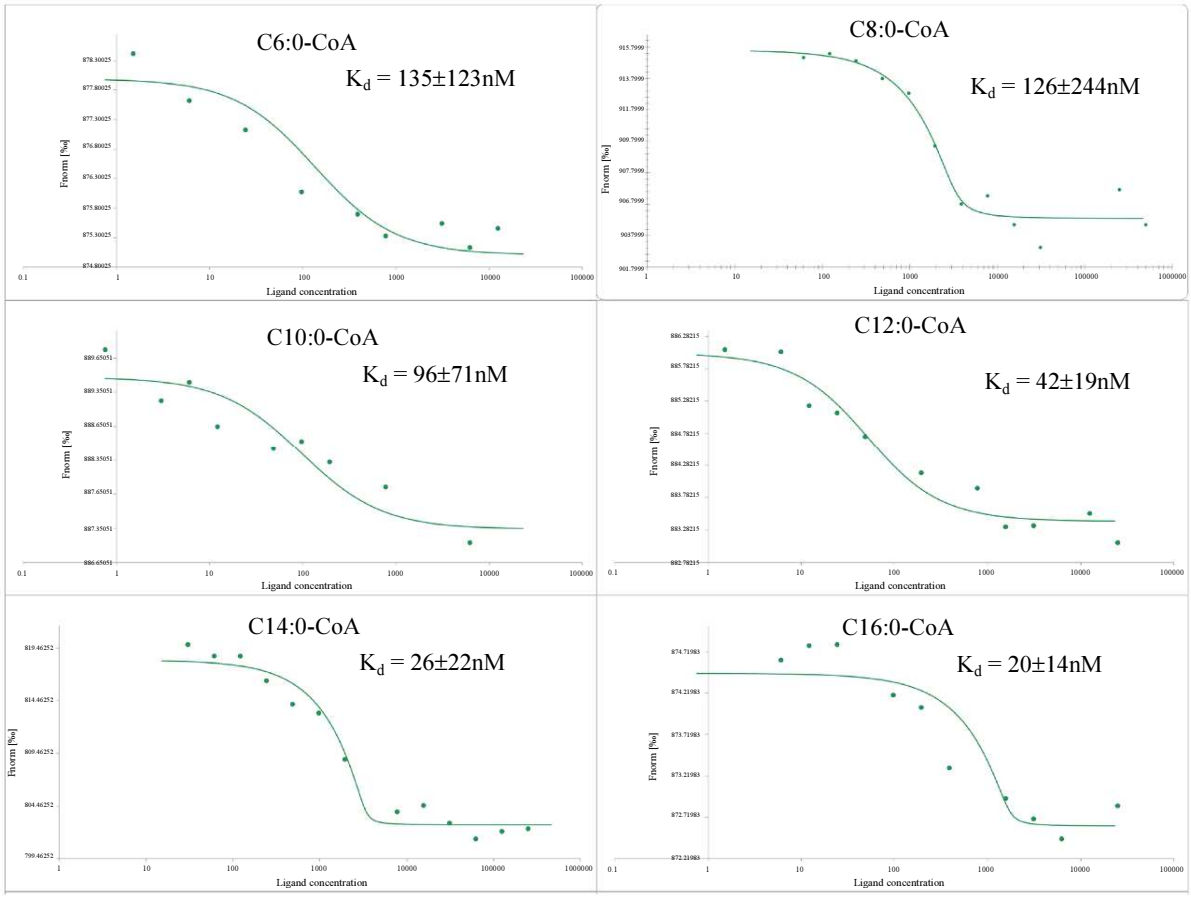


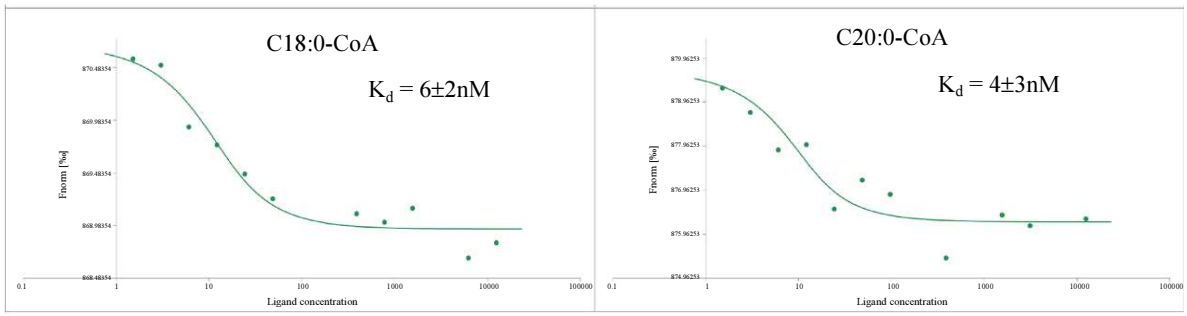


Fig. S1: Concentration response curve for His–AoAcbp1 ligand binding assessed by MST

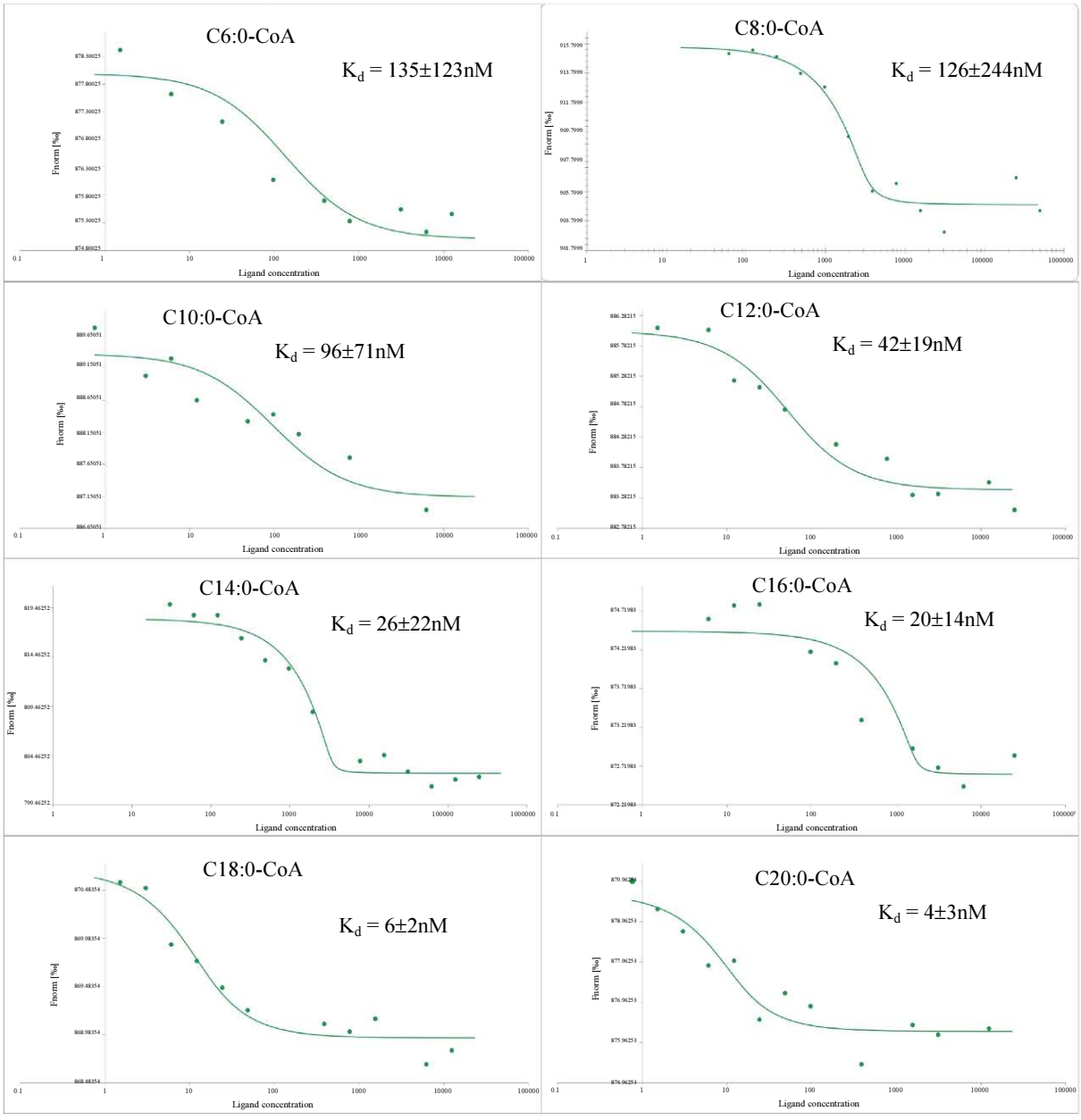


Fig. S2: Concentration response curve for MBP–AoAcbp1 ligand binding assessed by MST

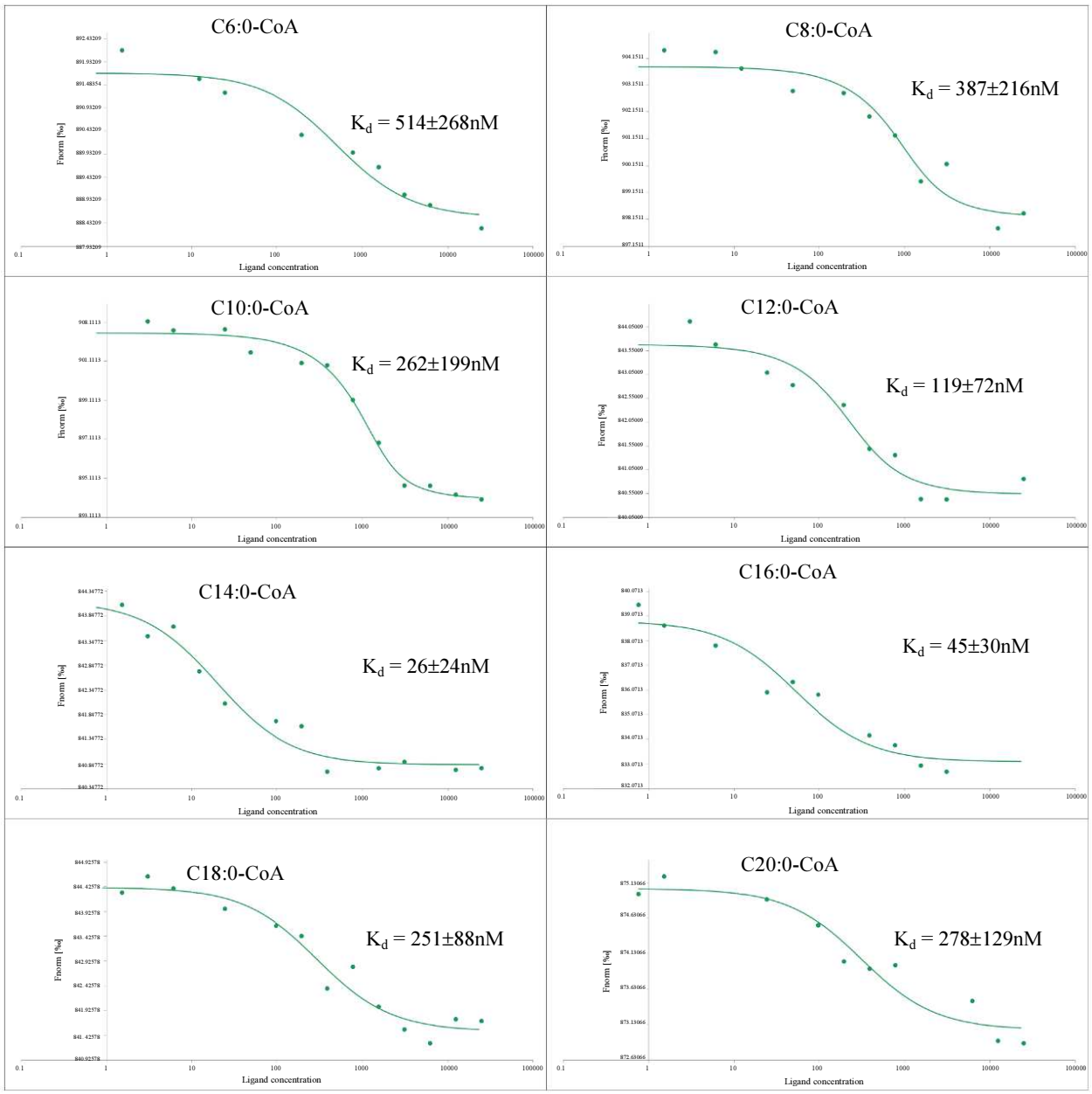
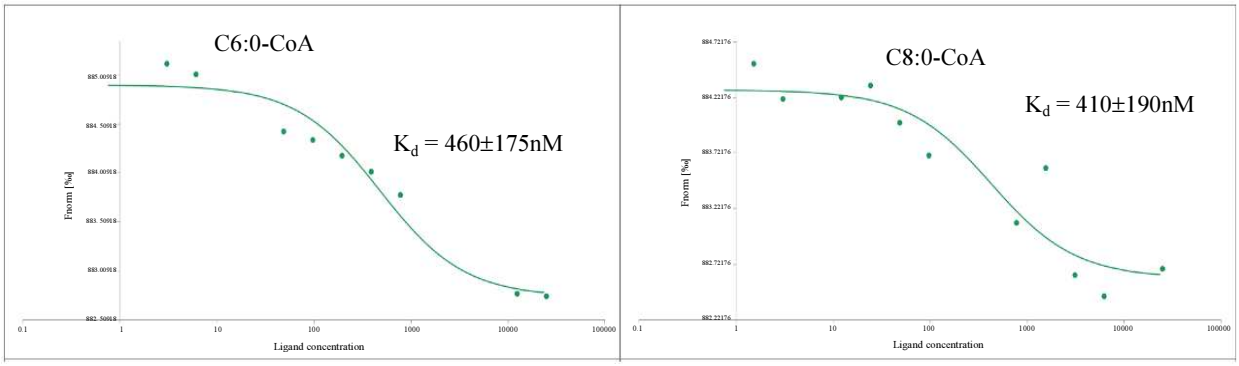


Fig. S3: Concentration response curve for His–ScAcbp ligand binding assessed by MST



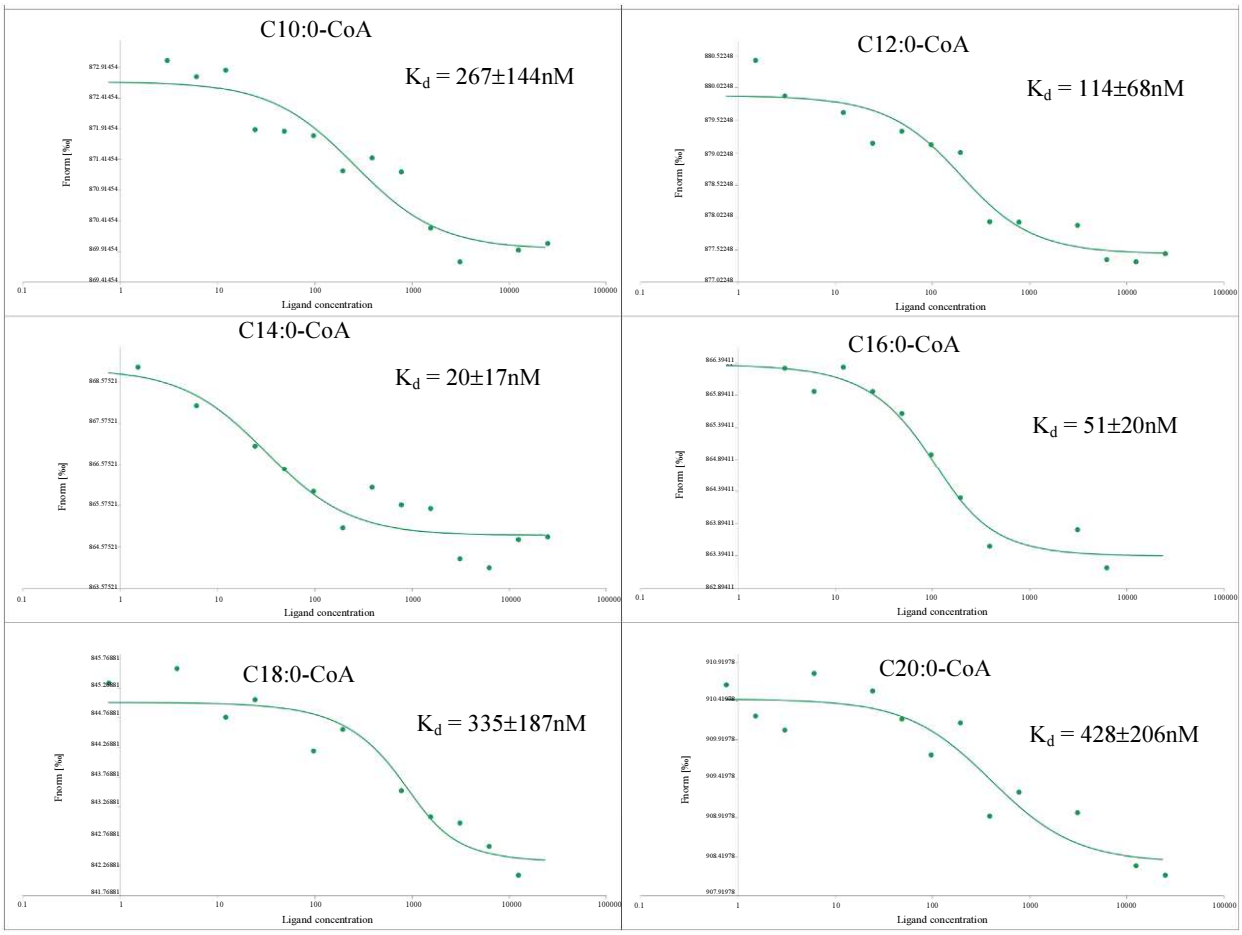


Fig. S4: Concentrationre sponse curve for MBP–ScAcbp ligand binding assessed by MST