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# Clone, expression and plasminogen binding property of three fructose-1,6-bisphosphate aldolases from *Clonorchis sinensis*

Li, S.[1,2,3#], Lu, B.P.[1#], Feng, J.[4], Zhou, J.J.[5], Xie, Z.Z.[6], Liang, C.[2,3], Li, X.R.[2,3], Huang, Y.[2,3] and Yu, X.B.[2,3*]
[1]School of Basic Medicine, Henan University of Chinese Medicine, Zhengzhou 450046, China
[2]Department of Parasitology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China
[3]Key Laboratory for Tropical Diseases Control of Ministry of Education, Sun Yat-sen University, Guangzhou 510080, China
[4]Zhengzhou YIHE Hospital Affiliated to Henan University, Zhengzhou 450047, China
[5]Zhengzhou Key Laboratory for Children's Infection and Immunity, Children's Hospital Affiliated to Zhengzhou University, Zhengzhou 450047, China
[6]Department of Clinical Laboratory, Third Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, China
[#]These authors contributed equally to this work.
[*]Corresponding author e-mail: yuxinbingteam@163.com
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**Abstract.** Fructose-1,6-bisphosphate aldolase (FbA), a well characterized glycometabolism enzyme, has been found to participate in other important processes besides the classic catalysis. To understand the important functions of three fructose-1,6-bisphosphate aldolases from *Clonorchis sinensis* (*Cs*FbAs, *Cs*FbA-1/2/3) in host-parasite interplay, the open reading frames of *Cs*FbAs were cloned into pET30a (+) vector and the resulting recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) for expression of the proteins. Purified recombinant *Cs*FbAs proteins (r*Cs*FbAs) were approximately 45.0 kDa on 12% SDS-PAGE and could be probed with each rat anti-r*Cs*FbAs sera by western blotting analysis. ELISA and ligand blot overlay indicated that r*Cs*FbAs of 45.0 kDa as well as native *Cs*FbAs of 39.5 kDa from total worm extracts and excretory-secretory products of *Clonorchis sinensis* (*Cs*ESPs) could bind to human plasminogen, and the binding could be efficiently inhibited by lysine analog ε-aminocaproic acid. Our results suggested that as both the components of *Cs*ESPs and the plasminogen binding proteins, three *Cs*FbAs might be involved in preventing the formation of the blood clot so that *Clonorchis sinensis* could acquire enough nutrients from host tissue for their successful survival and colonization in the host. Our work will provide us with new information about the biological function of three *Cs*FbAs and their roles in host-parasite interplay.

## INTRODUCTION

Clonorchiasis, closely related to cholangiocarcinoma and hepatocellular carcinoma, is a major public health problem in globally, especially some Asian countries such as China (including Hong Kong and Taiwan), Japan, Korea, and Vietnam (Li *et al*., 2020). *Clonorchis sinensis* (*C. sinensis*), the causative agent of clonorchiasis, has resulted in a serious socioeconomic burden in epidemic regions. Approximately 35 million people were infected with *C. sinensis* worldwide, including 15 million people in China (Lun *et al*., 2005). The molecular mechanism involved in clonorchiasis is very complex and incompletely clear. It is generally believed that excretory-secretory products (ESPs), continuously released or exfoliated proteins mixture from *C. sinensis*,

are toxic to and interact with host's bile epithelium (Pak *et al.*, 2009). Long-term stimulus from these molecules can provoke DNA mutation and chronic pathologic changes such as inflammation, epithelial hyperplasia, goblet cell metaplasia, periductal fibrosis, and biliary intraepithelial neoplasia (Sripa *et al.*, 2007). Since ESPs of *C. sinensis* (*Cs*ESPs) play a crucial role in host-parasite interaction, studies on them will be an essential step to further exploring the pathogenesis of clonorchiasis. Some proteins from *Cs*ESPs have been proven to participate in nutrients intake and energy metabolism, detoxification of bile components, and immune evasion (Wang *et al.*, 2011a; Huang *et al.*, 2012; Lei *et al.*, 2013; Liang *et al.*, 2013). However, the biological function of most proteins from *Cs*ESPs remains obscure to date.

Fructose-1,6-bisphosphate aldolase (FbA) is a ubiquitous and abundant glycometabolism enzyme that reversibly catalyzes D-fructose-1,6-bisphosphate to yield D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Maurady *et al.*, 2002). In our previous study, three FbA isozymes of *C. sinensis* (*Cs*FbAs, *Cs*FbA-1/2/3) were identified as the components of *Cs*ESPs, and two of the recombinant *Cs*FbAs (r*Cs*FbA-2 and r*Cs*FbA-3) showed catalytic activities (Li *et al.*, 2014). Recently, emerging evidences have demonstrated that some ancient metabolic enzyme, such as glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, and glycogen synthase kinases, can also participate in other important processes besides the classic catalytic activities, termed "moonlighting functions" (Nagini *et al.*, 2018; Sirover, 2018; Malhotra *et al.*, 2019; Rodriguez-Bolanos & Perez-Montfort, 2019). The "moonlighting functions" of metabolic enzymes are found throughout the archaea, bacteria, fungi, viruses, parasites, plants, insects, reptiles, fishes, birds, and even mammals. These metabolic enzymes play important roles in signal transduction (Kim *et al.*, 2002; Ishida *et al.*, 2005), vesicle trafficking and redistribution (Lu *et al.*, 2001; Lu *et al.*, 2004; Lu *et al.*, 2007; Merkulova *et al.*, 2011), cell mobility (Kusakabe *et al.*, 1997; Schindler *et al.*, 2001; Tochio *et al.*, 2010), as well as adhesion and invasion of the pathogen (Jewett & Sibley, 2003; Starnes *et al.*, 2009; Tunio *et al.*, 2010). It has been reported that metabolic enzymes of the pathogen present the "moonlighting functions" mostly relate to its virulence activity of the infection (Franco-Serrano *et al.*, 2018). Therefore, understanding the "moonlighting functions" of *Cs*FbAs will help us to find the molecular mechanisms of *C. sinensis* invading and infecting the host.

In the present study, three *Cs*FbAs were overexpressed in *Escherichia coli* (*E. coli*) BL21 (DE3) and their plasminogen binding properties were investigated. Our work will enrich the knowledge about the biological functions of *Cs*FbAs in host-parasite interplay and facilitate the explanation of the pathogenesis of clonorchiasis.

## MATERIALS AND METHODS

### Animals and parasites

Six-week-old male Sprague-Dawley (SD) rats were purchased from the animal center of Sun Yat-sen University. All animal experiments were approved by the Animal Care and Use Committee of Sun Yat-sen University (permit number SYXK (Guangdong) 2012-0081), and performed in accordance with Guangdong Provincial Laboratory Animal Administration Measures and the Laboratory Animal Administration Regulations issued by Ministry of Science and Technology of the People's Republic of China. Metacercariae of *C. sinensis* were isolated from experimentally infected *Pseudorasbora parva* in our ecologic pool (Liang *et al.*, 2009). The fish was digested with pepsin-HCl (pH 2.0) for 2 h at 37°C, and then filtered through the sieve mesh. The living metacercariae were washed several times with sterile phosphate-buffered saline (PBS) and collected under a light microscope. Adult worms were freshly collected from the bile ducts of SD rats infected with metacercariae for 8 weeks. After washing 3 times with sterile PBS, adult

worms were incubated in Sample Protecter (TaKaRa, Japan) and frozen in -80°C for RNA and protein extraction.

**Acquirement of recombinant *Cs*FbAs proteins (r*Cs*FbAs) and antisera**

The complete encoding sequences of *Cs*FbA-1, *Cs*FbA-2, and *Cs*FbA-3 (GenBank accession numbers: DF143094.1, DF143896.1, and DF143689.1) were obtained as described (Li *et al.*, 2014). Total RNA from *C. sinensis* adults was extracted with TRIZOL reagent (Invitrogen, USA) and the cDNA was synthesized by using the Reverse Transcriptase XL (TaKaRa, Japan). The open reading frames (ORF) of three *Cs*FbAs were amplified from cDNA of adult worms by using specific primers containing restriction enzyme sites as described (Li *et al.*, 2014). The purified PCR products were cloned into the 6× His tagged pET30a (+) vector (Novagen, USA). The resulting recombinant plasmids were identified by agarose gel electrophoresis and DNA sequencing, and then transformed into *E. coli* BL21 (DE3) (Promega, USA) for expression. The expression of recombinant proteins was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 4 h in Luria-Bertani medium. The induced cells were collected by centrifugation at 8,000*g* for 15 min at 4°C, suspended in lysis buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole; pH 8.0), and then sonicated for 20 min in an ice bath. The supernatants were obtained by centrifugation at 12,000*g* for 15 min at 4°C and purified by His Bind Purification Kit (Novagen, USA). The purified proteins were collected through gradient elution with 5-300 mM imidazole buffer and evaluated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Concentration of three purified r*Cs*FbAs was measured by BCA Assay Kit (Novagen, USA).

To obtain antisera, each rat was subcutaneously immunized with 200 μg r*Cs*FbA-1, r*Cs*FbA-2, or r*Cs*FbA-3 emulsified with equal volume of complete Freund's adjuvant (Sigma, USA). Two booster immunizations were performed at 2-week intervals with 100 μg r*Cs*FbA-1, r*Cs*FbA-2, or r*Cs*FbA-3 emulsified with equal volume of incomplete Freund's adjuvant (Sigma, USA). Anti-r*Cs*FbAs sera were obtained 2 weeks after the last immunization. Sera from unimmunized rats were also obtained and used as the control. Sera were stored at -80°C until use. Antibody titers were determined by enzyme-linked immunosorbent assay (ELISA).

**Western blotting analysis of r*Cs*FbAs and native *Cs*FbAs**

Worm extracts (WEs) and *Cs*ESPs were respectively prepared according to the previous method and their concentrations were measured by BCA Assay Kit (Novagen, USA) (Wang *et al.*, 2011a). Purified r*Cs*FbAs, WEs, and *Cs*ESPs were subjected to 12% SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% skim milk in PBS containing 0.05 % Tween 20 (PBS-T) at room temperature (RT) for 3 h, and then incubated with rat anti-r*Cs*FbAs sera or unimmunized sera (1:1,000 dilutions in PBS-T with 5% BSA) at 4°C overnight. After washing thoroughly, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG antibody (ProteinTech, USA; 1:10,000 dilutions) at RT for 1 h. Blots on the membranes were detected using an enhanced chemiluminescence (ECL) method (Millipore, USA).

**Plasminogen binding analysis of r*Cs*FbAs and native *Cs*FbAs**

Plasminogen binding properties of r*Cs*FbAs were determined by ELISA as described (de la Paz Santangelo *et al.*, 2011). Wells coated with 2 μg of purified r*Cs*FbA-1, r*Cs*FbA-2, r*Cs*FbA-3, fibrinogen (Calbiochem, Germany; positive control), or BSA (negative control) were respectively incubated with 50 μM human plasminogen (Athen, USA) in PBS-T with 1% BSA at 37°C for 2 h in the presence of 0, 50, 100, and 200 mM ε-aminocaproic acid (ε-ACA). In addition, wells coated with 2 μg of r*Cs*FbA-1, r*Cs*FbA-2, r*Cs*FbA-3, or BSA were incubated with 0-10.24 μM plasminogen, respectively. Rabbit anti-human plasminogen

polyclonal antibody (ProteinTech, USA; 1:5,000 dilutions) and HRP-conjugated goat anti-rabbit IgG antibody (1:20,000 dilutions) were used as the primary antibody and the secondary antibody, respectively. The reactions were visualized by TMB substrate solution (BD Biosciences) at OD 450 nm.

Ligand blot overlay assay was used to identify the binding property of r*Cs*FbAs and native *Cs*FbAs to human plasminogen according to the previous method (Lin *et al.*, 2011). Purified r*Cs*FbA-1, r*Cs*FbA-2, or r*Cs*FbA-3, as well as WEs and *Cs*ESPs were subjected to 12% SDS-PAGE and electro-transferred onto PVDF membranes. The membranes were blocked with PBS-T containing 5% skim milk at RT for 3 h, and then incubated with 50 μM human plasminogen in the presence or absence of 200 mM ε-ACA at 4°C overnight. After washing process, the membranes were incubated with rabbit anti-human plasminogen polyclonal antibody (1:5,000 dilutions) at 4°C overnight, and subsequently incubated with HRP-conjugated goat anti-rabbit IgG antibody (1:10,000 dilutions) at RT for 1 h. The blots on the membranes were detected by the ECL method.

**Statistical analysis**

Statistical analysis was performed using SPSS software 19.0. Data were represented as mean ± SD from at least 3 separate experiments.

## RESULTS

**Agarose gel electrophoresis identification of recombinant pET-30a (+)-*Cs*FbAs plasmids**

Recombinant three pET-30a (+)-*Cs*FbAs plasmids were respectively digested by restriction enzymes and identified by agarose gel electrophoresis, showing the bands of approximately 1,000 base pairs (bp) and 5,000 bp. The bands of 1,000 bp were in accordance with PCR amplification of three *Cs*FbAs ORFs (*Cs*FbA-1, 1089bp; *Cs*FbA-2, 1092bp; *Cs*FbA-3, 1092bp), while the bands of 5,000 bp were in accordance with pET-30a (+) digested by restriction enzymes (Figure 1A-C).

**Gradient elution of r*Cs*FbAs with imidazole buffer**

Imidazole eluent of r*Cs*FbAs was analyzed by SDS-PAGE for collection of the purified proteins. With the increasing concentration of imidazole, three r*Cs*FbAs were gradually purified, showing the bands of approximately 45.0 kDa on 12% SDS-PAGE. Purified r*Cs*FbA-1 were collected from 150, 200, and 300 mM imidazole eluent, purified r*Cs*FbA-2 were collected from 100 mM and 150 mM imidazole eluent, and purified r*Cs*FbA-3 were collected from 60, 80, and 100 mM imidazole eluent (Figure 2A-C).

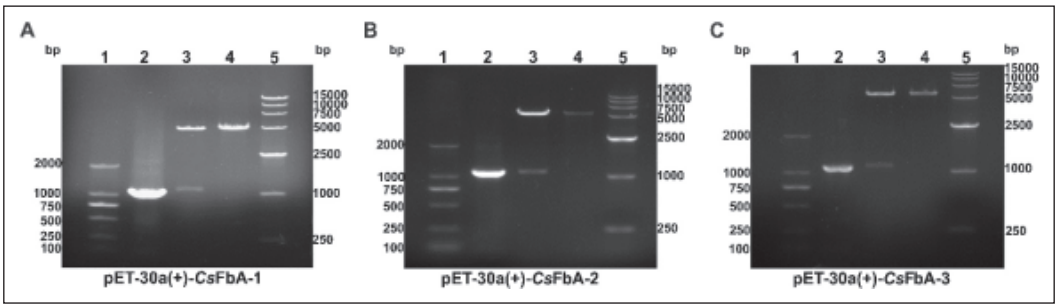


Figure 1. Agarose gel electrophoresis identification of recombinant pET-30a (+)-*Cs*FbAs plasmids. (A) pET-30a (+)-*Cs*FbA-1. (B) pET-30a (+)-*Cs*FbA-2. (C) pET-30a (+)-*Cs*FbA-3. DL2000 Maker (lane 1), PCR amplification products (lane 2), pET-30a (+)-*Cs*FbA-1, pET-30a (+)-*Cs*FbA-2 or pET-30a (+)-*Cs*FbA-3 digested by restriction enzymes (lane 3), pET-30a (+) digested by restriction enzymes (lane 4), and DL15000 Maker (lane 5).

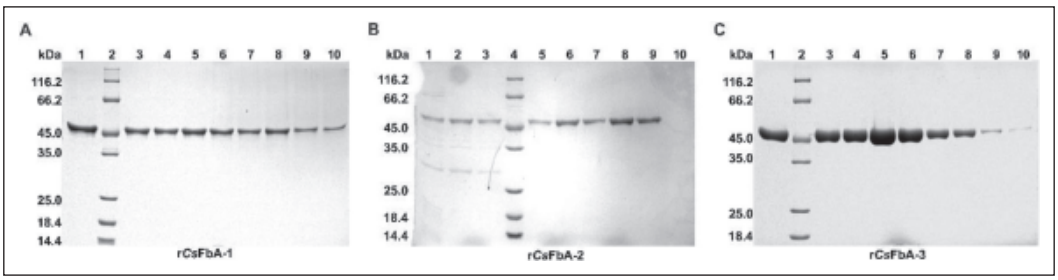


Figure 2. Gradient elution of r*Cs*FbAs with imidazole buffer. Imidazole eluent of r*Cs*FbAs was analyzed by SDS-PAGE for collection of the purified proteins. (A) r*Cs*FbA-1. Protein MV marker (lane 2), 150 mM imidazole eluent (lane 1, 3 and 4), 200 mM imidazole eluent (lane 5, 6 and 7), 300 mM imidazole eluent (lane 8, 9 and 10). (B) r*Cs*FbA-2. Protein MV marker (lane 4), 80 mM imidazole eluent (lane 1, 2 and 3), 100 mM imidazole eluent (lane 5, 6 and 7), 150 mM imidazole eluent (lane 8, 9 and 10). (C) r*Cs*FbA-3. Protein MV marker (lane 2), 60 mM imidazole eluent (lane 1, 3 and 4), 80 mM imidazole eluent (lane 5, 6 and 7), 100 mM imidazole eluent (lane 8, 9 and 10).

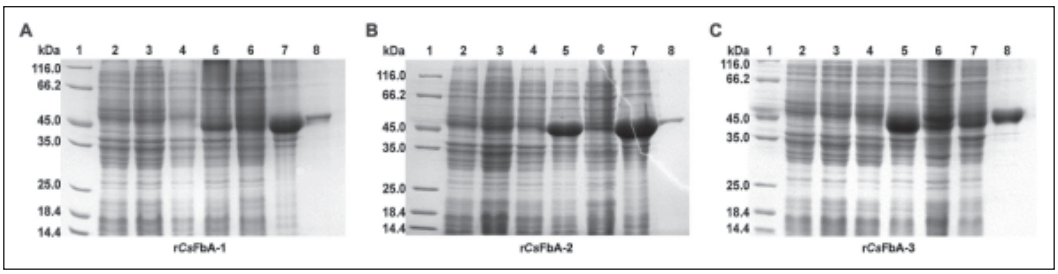


Figure 3. SDS-PAGE identification of r*Cs*FbAs. (A) r*Cs*FbA-1. (B) r*Cs*FbA-2. (C) r*Cs*FbA-3. Protein MV marker (lane 1), lysate of *E. coli* with pET-30a (+) before and after induction (lane 2 and 3), lysate of *E. coli* with pET-30a (+)-*Cs*FbA-1, pET-30a (+)-*Cs*FbA-2 or pET-30a (+)-*Cs*FbA-3 before and after induction (lane 4 and 5), supernatant and precipitation of induced pET-30a (+)-*Cs*FbA-1, pET-30a (+)-*Cs*FbA-2 or pET-30a (+)-*Cs*FbA-3 transformants (lane 6 and 7), and purified r*Cs*FbA-1, r*Cs*FbA-2 or r*Cs*FbA-3 (lane 8).

**SDS-PAGE identification of *Cs*FbAs**

Purified proteins of three r*Cs*FbAs with 6×His-tag were approximately 45.0 kDa confirmed by 12% SDS-PAGE, which were consistent with their theoretical molecular weight (MW; r*Cs*FbA-1, 45.0 kDa; r*Cs*FbA-2, 45.2 kDa; r*Cs*FbA-3, 45.2 kDa), including the extra amino acids from vectors (Figure 3A-C).

**ELISA assay of IgG titers from anti-*Cs*FbAs sera**

After rats immunized with r*Cs*FbAs, IgG antibody titers were assayed by ELISA. IgG titers in anti-r*Cs*FbA-1, anti-r*Cs*FbA-2, and anti-r*Cs*FbA-3 sera were respectively 1:102400, 1:204800, and 1:204800, indicating that high levels of IgG antibodies were obtained after rats immunized with r*Cs*FbAs (Figure 4A-C).

**Western blotting analysis of r*Cs*FbAs and native *Cs*FbAs**

Purified r*Cs*FbAs could be probed with each rat anti-r*Cs*FbAs sera at the band of approximately 45.0 kDa, while WEs and *Cs*ESPs could be probed with each rat anti-r*Cs*FbAs sera at the bands of approximately 39.5 kDa (MW of native *Cs*FbAs). However, purified r*Cs*FbAs, WEs, and *Cs*ESPs could not be probed with unimmunized sera (Figure 4D-F).

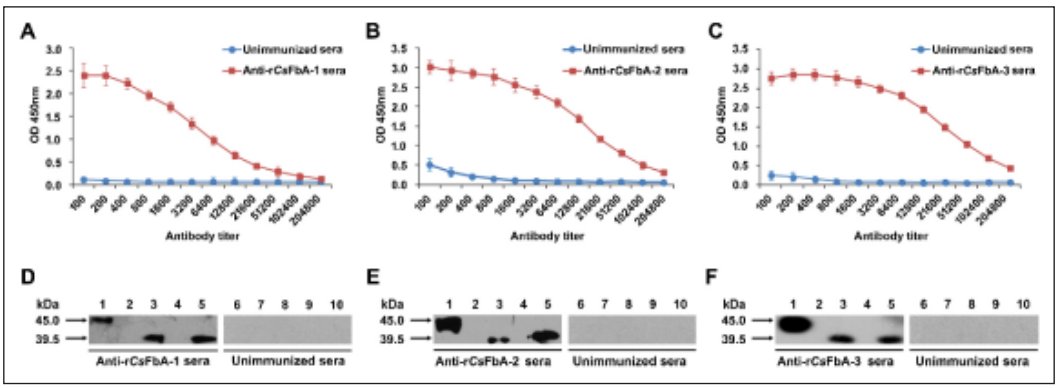


Figure 4. Western blotting analysis of r*Cs*FbAs and native *Cs*FbAs. IgG antibody titer in anti-r*Cs*FbA-1 sera (A), anti-r*Cs*FbA-2 sera (B) and anti-r*Cs*FbA-3 sera (C). P/N ≥ 2.1 was considered as an effective antibody titer (P, OD 450nm of anti-r*Cs*FbAs sera; N, OD 450nm of unimmunized sera). Western blotting analysis of r*Cs*FbA-1 (D), r*Cs*FbA-2 (E), r*Cs*FbA-3 (F), and native *Cs*FbAs from WEs or *Cs*ESPs. Purified r*Cs*FbA-1, r*Cs*FbA-2, or r*Cs*FbA-3 (lane 1 and 6), 1× loading buffer (lane 2 and 7), WEs (lane 3 and 8), 1× loading buffer (lane 4 and 9) and *Cs*ESPs (lane 5 and 10).

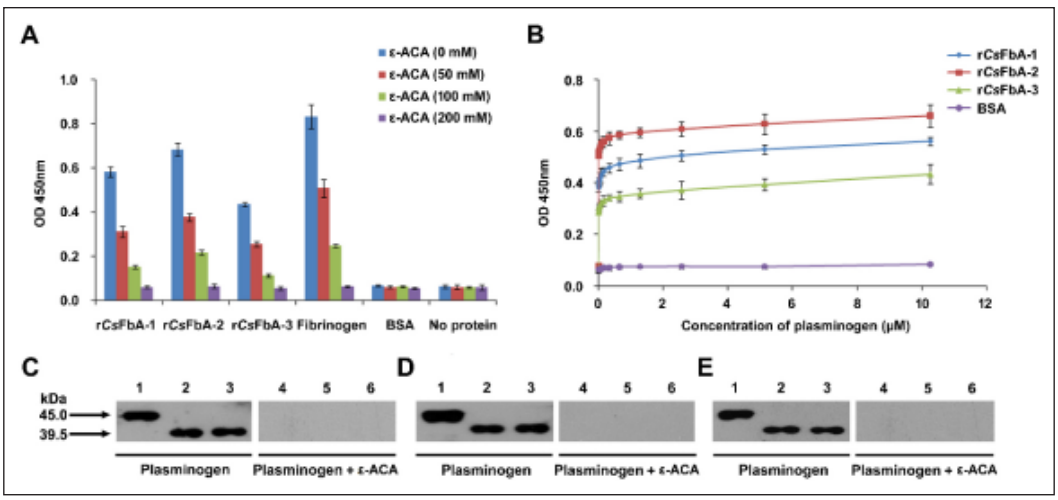


Figure 5. Plasminogen binding property of r*Cs*FbAs and native *Cs*FbAs. (A) Plasminogen binding property of r*Cs*FbAs assayed by ELISA. Wells coated with 2 μg of r*Cs*FbA-1, r*Cs*FbA-2, r*Cs*FbA-3, fibrinogen (positive control), or BSA (negative control) was incubated with 50 μM plasminogen in the presence of 0, 50, 100 and 200 mM ε-ACA. (B) Dose-dependent binding property of r*Cs*FbAs to plasminogen assayed by ELISA. Wells coated with 2 μg of r*Cs*FbA-1, r*Cs*FbA-2, r*Cs*FbA-3, or BSA (control) was incubated with 0-10.24 μM plasminogen. Plasminogen binding property of r*Cs*FbA-1 (C), r*Cs*FbA-2 (D), r*Cs*FbA-3 (E), and native *Cs*FbAs from WEs or *Cs*ESPs assayed by ligand blot overlay. Purified r*Cs*FbA-1, r*Cs*FbA-2, or r*Cs*FbA-3 (lane 1 and 4), WEs (lane 2 and 5), and *Cs*ESPs (lane 3 and 6).

### Plasminogen binding property of r*Cs*FbAs and native *Cs*FbAs

ELISA assay showed that the OD 450nm values of r*Cs*FbA-1, r*Cs*FbA-2, r*Cs*FbA-3, and fibrinogen were significantly higher than that of BSA when they were incubated with 50 μM human plasminogen in the absence of ε-ACA. The binding of three r*Cs*FbAs or fibrinogen to human plasminogen could be inhibited by 50, 100, and 200 mM lysine analogue ε-ACA (Figure 5A). In addition, the binding of three r*Cs*FbAs to plasminogen

exhibited a dose-dependent manner. With the increasing concentration of plasminogen (0-10.24 μM), the binding affinity of three r*Cs*FbAs was gradually strengthened. Apparently, r*Cs*FbA-2 protein showed the strongest plasminogen binding affinity, while r*Cs*FbA-3 protein showed the weakest plasminogen binding affinity (Figure 5B).

Ligand blot overlay assay showed that three r*Cs*FbAs incubated with human plasminogen could be blotted with rabbit anti-human plasminogen polyclonal antibody at the bands of approximately 45.0 kDa (MV of r*Cs*FbAs) in the absence of ε-ACA. WEs or *Cs*ESPs incubated with human plasminogen could also be blotted with rabbit anti-human plasminogen polyclonal antibody at the bands of approximately 39.5 kDa (MV of native *Cs*FbAs) in the absence of ε-ACA. However, neither r*Cs*FbAs nor WEs or *Cs*ESPs incubated with human plasminogen could be blotted with rabbit anti-human plasminogen polyclonal antibody in the presence of 200 mM ε-ACA (Figure 5C-E).

## DISCUSSION

FbA is a well-known glycometabolism enzyme responsible for energy production and metabolic processes in almost all organisms. Recently, it has been found to exert unusual "moonlighting functions" besides its glycometabolism activity (Shams *et al.*, 2014). To assess possible "moonlighting functions" of *Cs*FbAs, three isozymes were overexpressed in *E. coli* BL21 (DE3) and the purified proteins with expected MV of 45.0 kDa were obtained (Figure 1-3). FbA has been commonly identified as a molecule of tegument and ESPs from other trematodes including *Opisthorchis viverrini* (Mulvenna *et al.*, 2010), *Fasciola hepatica* (Jefferies *et al.*, 2001; Wilson *et al.*, 2011), *Schistosoma japonicum* (Liu *et al.*, 2009), and *Schistosoma bovis* (Perez-Sanchez *et al.*, 2006). In the present study, r*Cs*FbAs of 45.0 kDa and the native *Cs*FbAs of 39.5 kDa from ESPs and WEs could be blotted with anti-*Cs*FbAs sera by western blotting analysis, indicating that *Cs*FbAs were the components of *Cs*ESPs (Figure 4D-F). This result was consistent with the previous studies (Wang *et al.*, 2011b; Li *et al.*, 2014), implying that as the components of *Cs*ESPs, *Cs*FbAs could directly react with the bile duct of host and might play a moonlighting role in host-parasite interaction.

FbA has been proven to be a human plasminogen binding and activating protein in parasite (Ramajo-Hernandez *et al.*, 2007; Lorenzatto *et al.*, 2012) and non-parasite (Crowe *et al.*, 2003; Shams *et al.*, 2016). The phenomenon of human plasminogen binding is commonly found in bacteria (Boyle & Lottenberg, 1997), fungi (Crowe *et al.*, 2003), protozoa (Mundodi *et al.*, 2008; Gomez-Arreaza *et al.*, 2011; Figuera *et al.*, 2013), and helminth infection (Jolodar *et al.*, 2003; Bernal *et al.*, 2004; Ramajo-Hernandez *et al.*, 2007; de la Torre-Escudero *et al.*, 2010). These pathogens can utilize host plasminogen to generate proteolytic enzyme plasmin, which is responsible for degradation of the fibrin and the extracellular matrix, activation of the procollagenase, and release of the peptide for nutrition (Lahteenmaki *et al.*, 2001; Gomez-Arreaza *et al.*, 2011). In the present study, both r*Cs*FbAs as well as native *Cs*FbAs from ESPs and WEs could efficiently bind with human plasminogen by ELISA and ligand blot overlay assay (Figure 5A-E). As the components of *Cs*ESPs, the host plasminogen binding property of three *Cs*FbAs is probably beneficial for *C. sinensis* to invade and infect the host (Avilan *et al.*, 2011). During *C. sinensis* infection, the feeding and moving worms hook onto the biliary epithelium by their muscular suckers and continuous uptake blood from the small vessels of host for energy supply (Hou, 1955; Rim, 1986). Therefore, as both the components of *Cs*ESPs and the plasminogen binding protein, *Cs*FbAs might be involved in preventing formation of the blood clot so that the worms can acquire enough nutrients from the small vessels for their survival and colonization in the host. Plasminogen-receptor proteins possess carboxy-terminal lysine residues that may regulate their binding to plasminogen (Plow *et al.*, 1995). Lysine analogue ε-ACA can competitively inhibit plasminogen binding (Crowe *et al.*,

2003; Gomez-Arreaza *et al.*, 2011; Figuera *et al.*, 2013). In our study, plasminogen binding property of three r*Cs*FbAs could be effectively inhibited by lysine analog ε-ACA (Figure 5A and C-E), implying that *Cs*FbAs possibly contain carboxy-terminal lysine residues as the sites for plasminogen binding. Terminal lysine of *Neisseria meningitidis* FbA replaced by alanine did not completely block the binding and the residual binding was still blocked by lysine analog ε-ACA, suggesting that several lysine residues within FbA might contribute to the interaction with plasminogen (Shams *et al.*, 2016). Thus, it is speculated that plasminogen binding affinity of r*Cs*FbAs may depend on the density of terminal lysine sites. Further study will be necessary to identify the precise location of carboxy-terminal lysine residues in the amino acid sequences of three *Cs*FbAs.

In summary, three *Cs*FbAs was demonstrated to be of plasminogen binding property for the first time, suggesting that they might participate in preventing formation of the blood clot for adult worms to acquire enough nutrients from the host blood. Our results favored the speculation that three *Cs*FbAs possibly exert "moonlighting functions" besides its catalytic effect as a glycometabolism enzyme, which will help us to getting new information about the biological function of three *Cs*FbAs and their role in host-parasite interplay. Further studies will focus on the detailed "moonlighting functions" of three *Cs*FbAs participating in the pathophysiological processes of *C. sinensis* infection.

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**Conflict of Interests**

The authors declare that they have no competing interests.

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