NADH oxidase of Mycoplasma synoviae is a potential diagnostic antigen, plasminogen/fibronectin binding protein and a putative adhesin

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Abstract

Background

Mycoplasma synoviae (MS) is an important pathogen that causes respiratory diseases and arthritis in chickens and turkeys, resulting in serious economic losses to the poultry industry. Membrane-associated proteins were thought to play important roles in cytoadherence and pathogenesis. NADH oxidase (NOX) is a kind of oxidoreductase involved in glycolysis, which was thought to be a multifunctional protein and a potential virulence factor in some pathogens. However, there was still little knowledge about the NOX of MS (MSNOX). Our previous study has proved that the MSNOX was not only a cytoplasmic metabolic enzyme, but was also distributed in MS membrane. This study was mainly to explore the potential as a diagnostic antigen and an adhesion of the NOX in MS.

Results

In this study, Western blot analysis showed that recombinant MSNOX (rMSNOX) protein could react with positive sera against different MS isolates, but not MG isolates or other avian pathogens, suggesting the rMSNOX is a potential diagnostic antigen. In addition, the rabbit anti-rMSNOX serum showed a complement-dependent mycoplasmacidal rate as high as 86.5%. MS NOX protein has been identified as a cytoplasmic enzyme in our previous study, it was identified to be distributed on the surface of MS in this study using suspension immunofluorescence assays. Indirect immunofluorescence assays and colony counting assays showed that the rMSNOX and MS could adhere to DF-1 cells and the adherence could be significantly inhibited by the rabbit anti-rMSNOX serum. Moreover, plasminogen (Plg)- and fibronectin (Fn)-binding assays showed that rMSNOX was able to bind to Plg and Fn in a dose-dependent manner, further confirmed that MSNOX could be a putative adhesin.

Conclusion

The MSNOX was identified to be a surface immunogenic protein which also has good immunoreactivity and specificity, suggesting it may be used as a potential diagnostic antigen in future. The rMSNOX and MS presented adherence to DF-1 cells, which were inhibited by the rabbit anti-rMSNOX serum. Moreover, rMSNOX showed cytoadherence to extracellular matrix (ECM) proteins including Plg and Fn, suggesting that the NOX may play important roles in cytoadherence and pathogenesis of MS.

Background

Mycoplasma synoviae (MS) is a common poultry pathogen that leads to acute or chronic respiratory diseases, infectious synovitis, arthritis and eggshell apex abnormalities in chickens [1]. Usually, MS is considered to cause subclinical upper respiratory infection. When combined with Newcastle disease or infectious bronchitis, it can progress to respiratory with air sac disease, or to infectious synovitis when it

becomes systemic [2]. MS infection has been reported all over the world, and the epidemiological surveillance revealed that MS has a high prevalence rate in commercial chicken flocks [3–5]. Although the disease is rarely associated with mortality, it can cause significant economic losses to the poultry industry. In China, the seroprevalence of MS among 44,395 non-vaccinated chickens from 21 provinces from 2010 to 2015 has been reported to be 41.19% [6], which means the MS infection is widespread in China. It is necessary to clarify the pathogenesis of MS, which may help for further development of diagnostic antigens, subunit vaccines, and therapeutic drugs against MS infections.

Adherence is considered as the initial step for the pathogen to colonize and infect host cells, and the cytoadherence-related proteins may play important roles in pathogenesis. Due to lack of cell walls, the adherence of mycoplasma to host cells would mainly depend on the surface membrane-associated proteins. The most studied MS adhesin is the variable lipoprotein hemagglutinin (VlhA), which was identified as a highly divergent virulence-related factor [7–9]. In mycoplasmas, some glycolytic enzymes, such as enolase [10–13], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [14], the pyruvate dehydrogenase alpha and beta subunits (PdhA and PdhB) [15], have been identified as “moonlighting proteins”. They were proved not only to function as metabolic enzymes in cytoplasm, but also display on the surface of pathogen, and bind to host cells or host components.

NADH oxidase (NOX) in bacteria can catalyze the oxidation of NADH to NAD^+ by simultaneously reducing O_2 to H_2O or H_2O_2 [16, 17]. In Streptococcus, deletion or mutation of the *nox* gene can affect the bacteria growth, biofilm formation, competitiveness and virulence [18–21], which imply NOX is important for multiple biological functions. The *Mycoplasma bovis* (*M. bovis*) NOX was proved to function as both an active NADH oxidase and adhesin, and is therefore a potential virulence factor [22]. In our previous study, the MSNOX was confirmed to have the ability to oxidize NADH to NAD^+, and was distributed in both cytoplasm and membrane components of MS [23]. The membrane location of MSNOX protein suggests that it may participate in the interaction between MS and cell hosts. In this study, we further confirmed that NOX was surface localized on the MS cells, which could bind to host cells and extracellular matrix (ECM) proteins including plasminogen (Plg) and fibronectin (Fn). Moreover, the rabbit anti-rMSNOX serum exhibited significant adhesion inhibition activity. Those results further showed that the MSNOX plays an important role in the cytoadherence.

Results

Expression and immunological analysis of rMSNOX

The His-tagged recombinant MSNOX (rMSNOX) protein was expressed in *E. coli* BL21 and was then purified. The bacterial lysate supernatant and the purified rMSNOX protein were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A). Rabbit anti-rMSNOX serum was prepared and its antibody titers were detected to be 1:102400 by ELISA assay based on purified rMSNOX protein. Western blot assays showed the rMSNOX protein can be recognized by rabbit anti-rMSNOX serum, but not by the pre-immune rabbit serum (Fig. 1B), suggested the MSNOX was with good

immunogenicity. The immunoreactivity and specificity of MSNOX was also analyzed by Western blot assays. The results showed the purified rMSNOX protein could react with the positive chicken serum of different MS isolates (Fig. 1C, lanes 1–5), but not reacted with specific pathogen free (SPF) chicken serum (Fig. 1C, lane 6), the positive chicken sera of different *Mycoplasma gallisepticum* (MG) isolates (Fig. 1D, lanes 1–6), or positive sera against several other avian pathogens including *Mycoplasma iowae* (MI), *Escherichia coli* (*E. coli*) O1/O2/O78, *Salmonella pullorum/gallinarum* (SPG), Newcastle disease virus (NDV), infectious bursal disease virus (IBDV) and avian infectious bronchitis virus (IBV) (Fig. 1D, lanes 7–12).

Complement dependent mycoplasmacidal assay

Compared with pre-immune rabbit serum, rabbit anti-MS serum showed a mycoplasmacidal rate of 94.5% ($p < 0.01$), and the rabbit anti-rMSNOX serum presented a mycoplasmacidal rate of 86.5% ($p < 0.01$) (Table 1). These results indicated the obvious mycoplasmacidal activity of the rabbit anti-rMSNOX serum.

Table 1
Mycoplasmacidal activity of rabbit anti-rMSNOX serum

Sera	Mean CFU ± SD ($\times 10^3$)	Mycoplasmacidalrates (%)
Rabbit anti-MS serum	15.5 ± 1.8 [a]	94.5** [b]
Rabbit anti-rMSNOX serum	38.0 ± 9.9 [a]	86.5** [b]
Pre-immune rabbit serum	281.7 ± 3.6 [a]	-
[a] Results were from three replicate experiments.		
[b] Statistical significance compared to non-immunized rabbit serum were determined by Student's *t*-test (**, $p < 0.01$).		

Surface localization of NOX in MS

The suspension immunofluorescence assays showed obvious green fluorescence on the surface of MS by using rabbit anti-rMSNOX serum (Fig. 2A) or anti-MS serum (positive control) (Fig. 2B), while no green fluorescence was shown when using rabbit anti-rMSFBA (Fig. 2C, non-adhesion protein negative control) or pre-immune rabbit serum (Fig. 2D) as the primary antibody, which proved that the MSNOX protein existed on the surface of MS membrane.

Adherence and adherence inhibition of rMSNOX protein to DF-1 cells

The adherence of rMSNOX protein to DF-1 cells was identified by indirect immunofluorescence using rabbit anti-rMSNOX serum as the primary antibody and fluorescein isothiocyanate (FITC)-conjugate goat anti-rabbit IgG as the second antibody. Results showed that the DF-1 cells emitted significant green fluorescence on the surface when adhered by rMSNOX protein (Fig. 3A). The green fluorescence was inhibited by rabbit anti-rMSNOX serum (Fig. 3B), but not affected by pre-immune rabbit serum (Fig. 3C). Meanwhile, the cells treated with rMSFBA protein (Fig. 3D, non-adhesion protein negative control) or Dulbecco's modified eagle medium (DMEM, negative control) (Fig. 3E) showed very little or no green fluorescence on the surface, which proved that rMSNOX protein could adhere to DF-1 cells.

Adherence and adherence inhibition of MS to DF-1 cells

Colony counting assay was performed to determine the adherence and adherence inhibition abilities of MS to DF-1 cells. Adherence inhibition was performed by using rabbit anti-rMSNOX serum. MS pre-treated with rabbit anti-MS serum or pre-immune serum were used as the positive and negative control respectively. As was shown in Table 1, when compared to pre-immune rabbit serum, the adherence inhibition rates by rabbit anti-rMSNOX or anti-MS serum was 67.9 ($p < 0.01$) and 88.2% ($p < 0.01$) respectively (Table 2). This result indicated the MS adherence to DF-1 cells could be inhibited by rabbit anti-rMSNOX serum.

Table 2
Adherence inhibition rates of rabbit anti-rMSNOX serum

Sera	Mean CFU ± SD ($\times 10^3$)	Adherence inhibition rates (%)
Rabbit Anti-rMSNOX serum	16.6 ± 2.8 [a]	67.9** [b]
Rabbit anti-MS serum	6.1 ± 1.1 [a]	88.2** [b]
Pre-immune rabbit serum	51.8 ± 12.3 [a]	-

[a] Results were from three replicate experiments.

[b] Statistical significance compared to non-immunized rabbit serum were determined by Student's *t*-test (**, $p < 0.01$).

Chicken plasminogen (cPlg) and human fibronectin (hFn) binding ability of rMSNOX

The binding ability of rMSNOX to cPlg and hFn was confirmed by Western blot and ELISA assays. The Western blot assays showed both the cPlg (Fig. 4A) and hFn (Fig. 4B) could bind to rMSNOX protein with bands of about 53 kDa, and the binding ability was at dose-dependent pattern. No binding band was shown for bovine serum albumin (BSA) with cPlg/hFn under the same condition. Moreover, the ELISA assays were performed using plates coated with cPlg, hFn or BSA, and then incubated with serially

diluted rMSNOX protein. The results also confirmed that the rMSNOX protein was able to interact with cPlg (Fig. 4C) and hFn (Fig. 4D) in a dose-dependent pattern.

Discussion

The adherence of pathogenic microorganisms to host cells is an initial step for infection. Because mycoplasmas have no cell wall, the membrane proteins are considered to play an important role in the interaction between mycoplasma and host, and provide a place for this interaction [24]. The NOX in bacteria usually exists in the cytoplasm, plays an important role in regulating cell metabolism, such as maintaining the dynamic balance of NADH/NAD^+ in the process of glycolysis. In our study, the MSNOX was identified not only distributed in cytoplasm, but also existed in membrane components, prompting it may be involved in the process of cytoadherence. In *Streptococcus pneumoniae* (*S. pneumoniae*) [25] and *M. bovis* [22], the *nox*-deficient strain had a significant decrease in adhesion to host cells, proving that NOX may act as an adhesin. Indirect immunofluorescence assays showed that rMSNOX could adhere to DF1 cells, and the adherence could be significantly inhibited by rabbit anti-rMSNOX serum. In colony counting assay, the anti-rMSNOX serum caused 67.9% of adherence inhibition rates, predicting MSNOX plays an important role in MS cytoadherence.

Fn and Plg are widely known extracellular matrix proteins, they are common host cell factors that promote the interaction between pathogen and host cells [26–28]. Bacterial adherence to host tissues and ECM proteins is a critical step in the process of infection, since it establishes the initial contact with the host [29]. Fn plays important roles in several biological processes, such as adhesion to ECM, differentiation, growth and cell migration, and is considered to be the target of a large number of bacterial proteins [30]. The ability to bind to the Fn is a characteristic that has been reported for many pathogens [31]. In the study of the invasive capacity of *Staphylococcus aureus*, it appears that Fn-binding is a major virulence trait that enables this pathogen to cause invasive forms of disease and to persist within host cells [32]. Furthermore, beyond mediating bacterial adhesion, Fn has also been reported to participate in the inflammatory process by affecting leukocyte function and causes damage to the host [26]. In addition, Fn-binding proteins can be used as a means of targeted infection control, which have been investigated as potential antigens for vaccination [30]. Plg is the pro-enzyme of plasmin, the key enzyme of the fibrinolytic system. It mainly plays a regulatory role in fibrinolysis [33]. The plasmin (ogen) system is also known as a mediator of inflammation and the innate immune system [34]. Many pathogens, including the *Haemophilus influenza* [35], *Leptospira interrogans* [36], and *Aspergillus fumigatus* [37], were found to express or secret Plg-binding proteins to stimulate activation of Plg to plasmin. The plasminogen system is important for tissue remodeling and inflammation in addition to fibrinolysis. The plasmin generated by pathogenic activators or cofactors could directly degrade the fibrin or cleave the complement C3, C3b, and C5, which help impairing the chemotaxis, immune cell activation, phagocytosis, and membrane attack complex formation, thus enabling survival of the pathogen [34]. The ability to take advantage of plasminogen and its activated form plasmin is a common mechanism used by pathogenic bacteria in interaction with their respective host [38]. Some pathogenic microorganisms express proteins

that bind to and enhance the activity of plasminogen. In this way, pathogens utilize the host fibrinolytic system to promote invasion [39]. *Candida albicans* [40], *Cryptococcus neoformans* [39] and *Streptococcus* [38], all recruit plasminogen to further their own invasion and colonization in the host [41]. At present, some adhesion-related proteins were identified to bind to Plg or Fn [10, 15, 42]. In this study, we showed that rMSNOX could bind to cPlg and hFn in a dose-dependent fashion, which suggests that MSNOX may play an important role in the adhesion and invasion of MS to host cells, thus playing a pathogenic role. In the *S. pneumoniae* [43] and *S. suis* [44], NOX was proved to be an essential factor for infection, and the virulence of NOX mutant was also significantly reduced. The precise mechanism by which the NOX is involved in the pathogenesis of MS remains to be explored.

Due to the widespread prevalence of MS infection and its large economic impact on the chicken industry, it is particularly important to establish an accurate and effective diagnostic method. For detection of MS antibodies, the major membrane protein MSPB has been used as coating antigen, and was thought to be a specific and sensitive diagnostic antigen [45, 46]. However, the MSPB contains a proline-rich repeat region, which is prone to insertion or deletion mutation, and resulting in antigenic variation [47, 48]. Therefore, screening a sensitive, specific and highly conserved antigen is important. In this study, the rMSNOX could strongly react with the positive chicken sera against different MS isolates (including MS WVU_{1853}, JS1, HB1, SD1, and SH1) at 53 kDa, confirming the NOX was a sensitive and conserved immunoreactive protein in MS species. Besides that, the rMSNOX protein did not react with the SPF chicken serum, the positive chicken sera of MG isolates and some other major avian pathogens, suggesting the rMSNOX has good specificity. It is the first time to find that NOX protein has potential as a diagnostic antigenic target for MS antibody detection, and further study remains to be done.

The mice immunized with rNOX elicited a protective immune response to intranasal or intraperitoneal *S. pneumoniae* challenge, which renders NOX as a candidate for future pneumococcal vaccine [25]. In our study, immunogenicity analysis showed the rMSNOX was an immunogenic antigen. Since rabbit anti-rMSNOX serum can significantly inhibit the adherence of MS to DF-1 cells, and the antiserum had an obvious bactericidal effect (86.5%) in the presence of complement, we propose that MSNOX may also be used as an immunoprotective antigen for subunit vaccine in the future. However, the vaccine potential for rMSNOX requires further validation.

Conclusion

The rMSNOX was confirmed as a surface-exposed immunogenic protein that could bind to DF-1 cells and the ECM proteins including cPlg and hFn. Besides, rabbit anti-rMSNOX serum can significantly inhibit the adhesion of the rMSNOX to DF-1 cells, also can effectively inhibit the adhesion between MS and DF-1 cells. Furthermore, the good immunoreactivity and specificity of rMSNOX with MS-positive sera generated by different strains and other sera suggests that it can be used as a candidate for future diagnostic antigen. This study will help us to further understand the biological function of MSNOX protein and its role in the pathogenesis of MS.

Methods

Bacterial strains, plasmids, cell line, and chicken positive sera against different avian pathogens

The MS WVU_{1853} was purchased from the China Veterinary Culture Collection Center (CVCC, Beijing, China) and cultured in Mycoplasma Broth Base (Hopebio, Qingdao, China) supplemented with 0.01% Nicotinamide adenine dinucleotide (NAD) (Roche, Shanghai, China) and 10% porcine serum (Gibco, Carlsbad, CA, USA) at 37°C in an atmosphere of 5% CO_2. Contiguous cell lines of chicken embryo fibroblasts DF-1 were purchased from Shanghai Institute of Biochemistry and Cell Biology and cultured in DMEM supplemented with 10% fetal bovine serum. Chicken positive sera against different MS strains (including MS WVU_{1853}, MS JS1, MS HB1, MS SD1, and MS SH1), and against different MG strains (including MG R_{low}, MG 08 MG 013 MG FBH MG SGN MG SS) were prepared in our lab as described previously [49]. The other chicken positive sera against MI, *E. coli* O1/O2/O78, SPG, NDV, IBDV and IBV were all obtained from CVCC.

Expression and purification of rMSNOX

The His-tagged rMSNOX protein was expressed and purified from recombinant strain *E. coli*BL21 (pET28a-MS*nox*) as described previously [23]. The purified rMSNOX protein was analyzed by 12.5% SDS-PAGE and the protein concentration was detected by BCA protein assay kit (Beyotime, Beijing, China).

Preparation of rabbit anti-rMSNOX and anti-MS serum

Four two-month-old New Zealand white rabbits were purchased from Songlian experimental animal farm (Songjiang District, Shanghai, China) and pre-immune serum was collected as a negative control. To prepare polyclonal antibody against rMSNOX, the rabbits were injected subcutaneously 3 times at 2-week intervals with 300 μg of purified rMSNOX protein, or 10^9 color change unit (CCU) inactivated MS whole cells mixed with an equal volume of Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA), respectively. Two weeks after the third immunization, blood samples from immunized rabbits were collected to separate antiserum. The antibody titers of the antiserum against rMSNOX or MS were analyzed by ELISA assay using plates coated with purified rMSNOX protein (0.5 μg/well) or MS whole cells (10^7 CCU/well), respectively.

Immunogenicity, immunoreactivity and specificity analysis of rMSNOX

The purified rMSNOX protein (0.5 μg/well) was subjected to SDS-PAGE followed by transferring to nitrocellulose filter (NC) membrane. The NC membrane was then blocked with 5% skimmed milk at 37°C for 2 h and incubated with rabbit anti-MSNOX serum (1:1000) or pre-immune rabbit serum (1:1000) at 37°C for 1.5 h, respectively. After washing 3 times by PBST (PBS adding 0.05% Tween-20), the NC membranes were incubated with horseradish peroxidase (HRP) labeled goat anti-chicken IgY antibody

(1:5000; Abbkine, Redlands, CA, USA) at 37°C for 1 h. Then the membranes were visualized using an enhanced chemiluminescet (ECL) substrate kit (Yeasen, Shanghai, China).

In order to evaluate the immunoreactivity of rMSNOX with different MS-positive chicken sera, the positive sera against different MS strains (including MS WVU_{1853}, JS1, HB1, SD1, and SH1; 1:500) and goat anti-chicken IgY-HRP antibody (1:5000; Abbkine) were used to probe rMSNOX proteins (0.5 μg/well) by Western blot assays, as described above.

The specificity of rMSNOX was also assessed by Western blot assays as described above. The rMSNOX protein (0.5 μg/well) was used to react with chicken sera against other avian pathogens, including different MG strains (R_{low}, 08, 013, FBH, SGN, SS), *E. coli* O1/O2/O78, SPG, NDV, IBDV and IBV (each diluted 1:500), the SPF chicken serum (CVCC; 1:500) was used as the negative control.

Complement dependent mycoplasmacidal assay

Mycoplasmacidal assays were performed as described previously with some modifications [50]. All the rabbit sera were inactivated at 56°C for 30 min before use. MS WVU_{1853} were cultured to mid-logarithmic phase, washed 3 times with PBS by centrifuged at 5000 g for 15 min at 4°C, and resuspended with DMEM. The reaction mixture containing 30 μL of MS bacteria suspension [4.6×10^8 Colony-forming units (CFU)/mL)] and 10 μL of rabbit anti-MSNOX or anti-MS serum, or pre-immune rabbit serum was incubated at 37°C for 30 min. Then, 10 μL of complement (CVCC) was added and mixed thoroughly. After incubation at 37°C for 1 h, the reaction mixture was ten folds gradient diluted in mycoplasma broth and spread onto solid medium to count the colonies. Rabbit anti-MS serum and pre-immune rabbit serum were regarded as positive and negative controls, respectively. Three independent experiments were repeated and the mycoplasmacidal rates were calculated according to the following formula: [(CFU of pre-immune serum treatment - CFU of antiserum treatment) / (CFU of pre-immune serum treatment)].

Suspension immunofluorescence assay

The NOX has been identified to distribute in both cytoplasm and cell membrane components of MS in our previous study [23]. To determine the surface localization of NOX in MS, suspension immunofluorescence assay was performed as previously described.

MS bacteria were collected by centrifugation at 12000 g and washed twice with phosphate buffer saline (PBS). Then the MS cells were re-suspended and incubated with rabbit anti-rMSNOX serum (1:1000 diluted by PBS) at 37°C for 1 h. The rabbit anti-MS serum (1:1000) was used as a positive control, the rabbit anti-rMSFBA serum and pre-immune rabbit serum (1:1000) were used as negative controls. The fructose-1,6-bisphosphate aldolase (FBA) was previously identified as a cytoplasmic protein in MS [51].After washing three times with PBST, the MS cells were incubated with FITC-conjugate goat anti-rabbit IgG (1:1000, Sigma-Aldrich) at 37°C for 2 h. After washing, the MS pellets were spread onto the glass slides and observed by fluorescence microscope (Ni-U; Nikon, Tokyo, Japan).

Adherence and adherence inhibition assays

To detect the adherence and adherence inhibition of rMSNOX to DF-1 cells, indirect immunofluorescence assays were performed as described previously, with some modifications [11]. The DF-1 cells were propagated on coverslips in 12-well cell culture plates for 24 h. After washing, the DF-1 cells were incubated with 10 μg of freshly purified rMSNOX or rMSFBA protein in 500 μL DMEM for 1 h at 37°C. The DF-1 cells treated with His-tagged rMSFBA protein or DMEM were used as negative or blank control [52]. For the adherence inhibition assay, the rMSNOX was pre-incubated with rabbit anti-rMSNOX serum or pre-immune rabbit serum (1:50 diluted by PBS) for 1 h at 37°C before adding it to the blocked cells. After washing 4 times with PBST to remove the unadhered protein, the bound rMSNOX or rMSFBA protein was recognized by rabbit anti-rMSNOX or rabbit anti-rMSFBA serum (1:1000) for 1 h, and was then labeled with goat anti-rabbit IgG-FITC (1:1000, Sigma-Aldrich) for 1 h. The cell membranes and nuclei were stained with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanineperchlorate (Dil, Beyotime) and 4',6-diamidino-2-phenylindole (DAPI, Beyotime), according to methods described previously [10]. Finally, the cellular coverslips were treated with antifade mounting medium (Sangon Biotech, Shanghai, China) and observed under a laser scanning confocal microscopy (LSM800; Zeiss, Oberkochen, German).

The adherence inhibition of MS to DF-1 cells by anti-rMSNOX serum was estimated by colony counting assays. DF-1 cells were propagated in DMEM on microscope coverslips in 6-well cell culture plates for 24 h. The MS WVU_{1853} cultured to logarithmic growth phase was collected by centrifugation at 5000 g for 15 min and was then incubated with rabbit anti-rMSNOX (1:50), anti-MS (1:50) or pre-immune serum (1:50) at 37°C for 1 h, respectively. The rabbit anti-MS and pre-immune serum was used as positive and negative controls. All the rabbit sera were inactivated at 56°C before use. The DF-1 cells were infected with the pre-treated MS cells at 200 multiplicity of infection (MOI) at 37°C for 2 h, and then were washed thoroughly with PBS to remove the un-adhered mycoplasma. After that, the cells were lysed with 1 mL serum-free Mycoplasma culture medium for 20 min and then the cell suspension was collected. The suspension was serially diluted and spread onto mycoplasma agar plates for colony counting. Three independent experiments were performed in triplicate. The adherence inhibition rate was calculated as: [(CFU from pre-immune serum treatment - CFU from antiserum treatment) / CFU from pre-immune serum treatment] × 100%.

Binding activities of rMSNOX to cPlg and hFn

Western blot and ELISA were used to determine the binding activities of rMSNOX to cPlg and hFn.

For western blot analysis, gradiently diluted rMSNOX protein (2, 1, and 0.5 μg) and 2 μg BSA were subjected to 12.5% SDS – PAGE, and then transferred to NC membrane. After blocking with 5% skimmed milk, the NC membrane was incubated with 10 μg/mL of cPlg (Cell Sciences, Canton, MA, USA) or hFn (Sigma-Aldrich) for 2 h at 37℃. After excessive washing with PBST, the membrane was incubated with rabbit anti-cPlg/hFn polyclonal antibody (1:1000; Cell Sciences) for 1 h at 37°C, followed by incubation with HRP conjugated goat anti-rabbit IgG antibody (1:5000; Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at 37°C. Membranes were visualized using an enhanced ECL substrate kit (Yeasen).

For ELISA analysis, the 96-well plates were coated with 1 μg/well of cPlg (Cell Sciences, Canton, MA, USA) or hFn in carbonate coating buffer (2.94 g/L $NaHCO_3$, 1.6 g/L Na_2CO_3, pH 9.6) at 37°Cfor 2 h. Wells coated with 1 μg/wellof BSA (Sigma-Aldrich) were used as negative controls. After washing three times with PBST, wells were blocked with 5% no-fat milk in PBST and then incubated with different amount of rMSNOX protein (1, 0.5, 0.15, 0.125, 0.0625, and 0.03125 μg/well) at 37°Cfor 1.5 h. After washing, the wells were treated with rabbit anti-rMSNOX serum (1:500) at 37°C for 1 h, followed by HRP conjugated goat anti-rabbit IgG antibody (1:5000; Thermo) at 37°C for 1 h. Each well was added with 100 μL of soluble TMB substrate solution (Tiangen) and incubated for 15 min for color reaction, which was then terminated by 50 μL of 2 M H_2SO_4. Finally, the absorbance values at 450 nm were measured by a spectrophotometer (SynergyH1, Biotek). The experiments were performed in triplicate.

Statistical analysis

Data are given as mean ± standard deviation (SD) for three replicate experiments, and the statistical analyses were performed using Student's t-test. Significant differences were denoted as $^{*}p < 0.05$ and $^{**}p < 0.01$.

Abbreviations

MS: *Mycoplasma synoviae*, NOX: NADH oxidase, MSNOX: NOX of *Mycoplasma synoviae*, rMSNOX: Recombinant MSNOX, Plg: Plasminogen, Fn: Fibronectin, ECM: Extracellular matrix, VlhA: Variable lipoprotein hemagglutinin, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, PdhA and PdhB: Pyruvate dehydrogenase alpha and beta subunits, *M. bovis: Mycoplasma bovis*, SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SPF: specific pathogen free, MG: *Mycoplasma gallisepticum*, *E. coli*: *Escherichia coli*, SPG: *Salmonella pullorum/ gallinarum*, NDV: Newcastle disease virus, IBDV: Infectious bursal disease virus, IBV: Avian infectious bronchitis virus, FITC: Fluorescein isothiocyanate, DMEM: Dulbecco's modified eagle medium, CPlg: Chicken plasminogen, HFn: Human fibronectin, BSA: Bovine serum albumin, *S. pneumoniae*: *Streptococcus pneumoniae*, NAD: Nicotinamide adenine dinucleotide, CCU: Color change unit, NC: Nitrocellulose filter, HRP: Horseradish peroxidase, ECL: Enhanced chemiluminescence, CFU: Colony-forming units, PBS: phosphate buffer saline, FBA: Fructose-1,6-bisphosphate aldolase, DiI: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate, DAPI: 4',6-diamidino-2-phenylindole, MOI: Multiplicity of infection, SD: Standard deviation.

Declarations

Ethics approval and consent to participate

The animal experiments were performed in strict accordance with the guidelines of the Care and Use of Laboratory Animals of Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences (CAAS). All animal experimental procedures were approved by the Committee on the Ethics of Animal Experiments of Shanghai Veterinary Research Institute, CAAS (Permit Number: SHVRI-ra-20180803-02). The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article. Further inquiries can be directed to the corresponding authors.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZJH and HRL performed most of the experiments and drafted the manuscript. YBS and YTC prepared the antiserum against rMSNOX and helped for immunoreactivity detection. YXZ participated in cultivation of Mycoplasma and preparation of fresh rMSNOX protein. SHW and MXT helped to revise the manuscript. WGJ contributed in experiment design and manuscript revision. JJQ and SQY conceived the study, designed the experiment, revised and finalized the manuscript. All authors read and approved the final manuscript.

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Figures

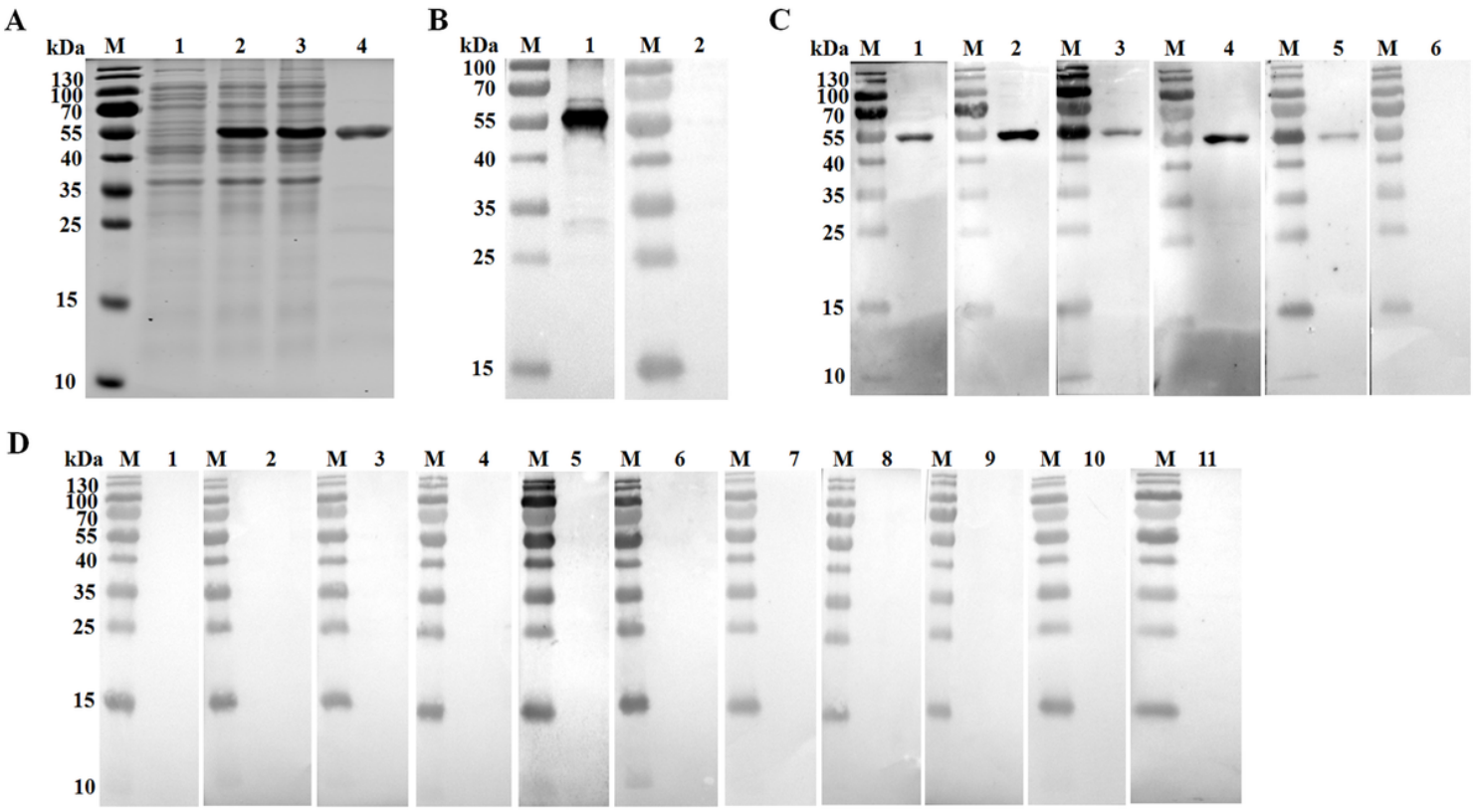

Figure 1

Expression and immunological characteristics of rMSNOX protein. (A) SDS-PAGE analysis of expression and purification of rMSNOX protein. Lane M: protein marker, lane 1: cell lysates of *E. coli* BL21 containing

empty vector, lane 2: total cell lysates of recombinant strain *E. coli* BL21 (pET28a-MS*nox*), lane 3: supernatant of total cell lysates of recombinant bacteria, lane 4: purified His-tagged MSNOX protein. The gel in this figure was cropped and the original gel was presented in Supplementary Fig. S1. (B) Immunogenicity analysis of rMSNOX protein. Lane M: protein marker, lane 1: Western blot analysis of the purified rMSNOX using rabbit anti-rMSNOX serum (1:1000), lane 2: Western blot analysis of the purified rMSNOX using pre-immune rabbit serum (1:1000). The blots were divided with white space, which were cropped from two different blots under the same exposure. The original blots are presented in Supplementary Fig. S2. (C) Immunoreactivity analysis of rMSNOX using Western blots. Lanes 1-5: positive chicken serum against different MS isolates (MS WVU_{1853}, MS JS1, MS HB1, MS SD1, and MS SH1), lane 6: negative serum from SPF chicken. (D) Specificity analysis of rMSNOX using Western blots. Lanes 1-6: positive chicken sera of different MG isolates (MG R_{low}, MG 08, MG 013, MG FBH, MG SGN, MG SS), lanes 7-12: positive sera against other avian pathogens (MI, *E. coli* O1/O2/O78, SPG, NDV, IBDV and IBV). All of the chicken sera were diluted at 1:500. The grouping of blots were cropped from different gels and were divided with white space. The samples derive from the imunoblot experiments and the gels/blots were processed in parallel. Full-length blots for Fig.1 C and D are presented in Supplementary Fig. S3.

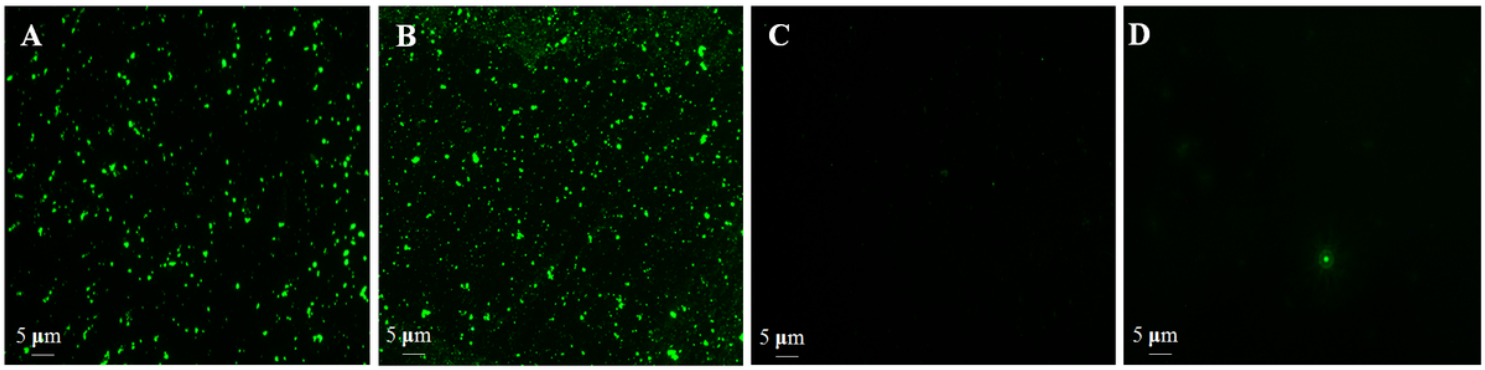

Figure 2

Suspension immunofluorescence assays. MS cells were treated with rabbit anti-rMSNOX serum (A), rabbit anti-MS serum (B), rabbit anti-rMSFBA serum (C), or pre-immune rabbit serum (D), followed by goat anti-rabbit IgG-FITC antibody.

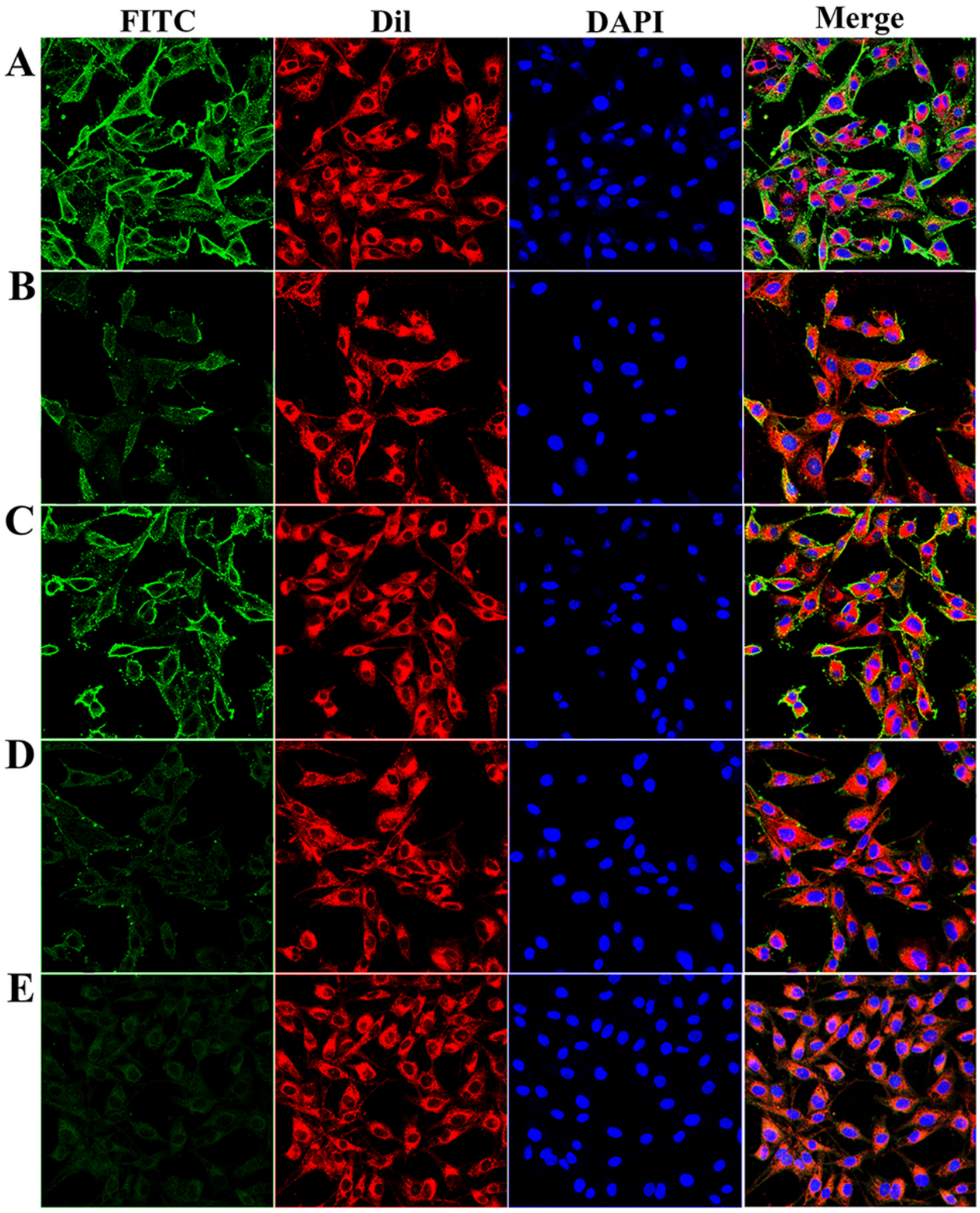

Figure 3

Indirect immunofluorescence assays to detect the adherence and inhibition of rMSNOX to DF-1 cells. (A) Adhesion of rMSNOX to DF-1 cells. DF-1 cells were incubated with 10 μg of rMSNOX protein and then were labeled by rabbit anti-rMSNOX serum and goat anti-rabbit IgG-FITC antibody (Green). The DF-1 cell membranes and nuclei were stained with Dil (red) and DAPI (blue) respectively. (B) Adhesion of anti-serum pre-treated rMSNOX protein to DF-1 cells. The adherence was significantly inhibited by rabbit anti-

rMSNOX serum. (C) Adhesion of pre-immune serum treated rMSNOX protein to DF-1 cells. Treatment with pre-immune rabbit serum showed no effect on adhesion. (D) Adhesion of rMSFBA protein to DF-1 cells (Negative control). The rMSFBA protein were then labeled with rabbit anti-rMSFBA serum and goat anti-rabbit IgG-FITC antibody (green). Little or no green fluorescence showed on the surface of DF-1 cells. (E) DF-1 cells treated with DMEM without protein (Blank control).

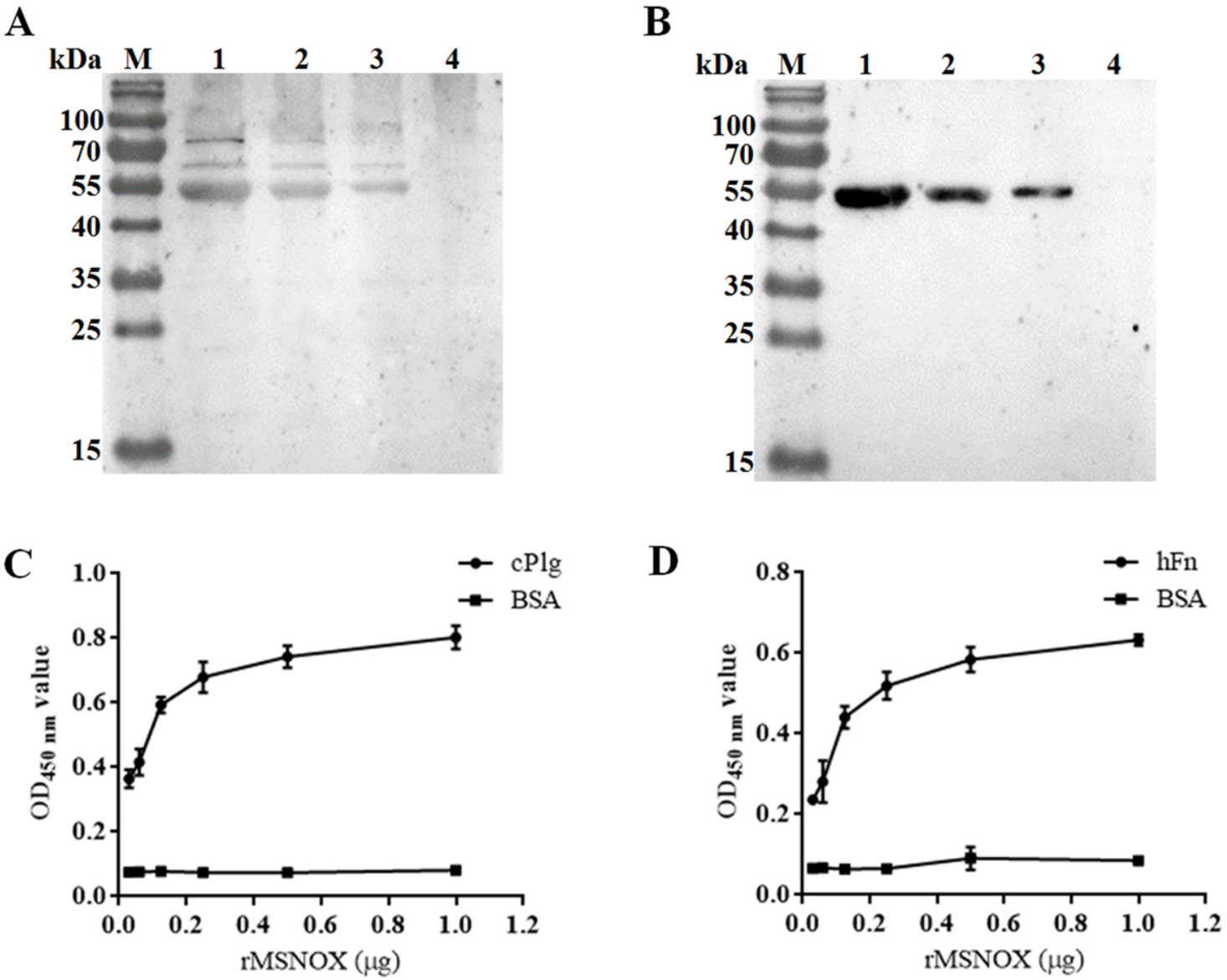

Figure 4

Determination of rMSNOX binding to cPlg and hFn. The binding abilities of rMSNOX to cPlg (Fig. 4A) and hFn (Fig. 4B) were confirmed by Western blot assays. Gradiently diluted rMSNOX protein (lanes 1-3: 2, 1, and 0.5 μg) and 2 μg BSA (lane 4) were incubated with 10 μg/mL of cPlg or hFn, and then recognized by rabbit anti-cPlg or anti-hFn polyclonal antibody (1:1000). The full-length figures are presented in Supplementary Fig. S4. Binding abilities of rMSNOX to cPlg (Fig. 4C) and hFn (Fig. 4D) were also identified by ELISA assays using rabbit anti-rMSNOX serum (1:500). Plates were coated with 1 μg of cPlg

or hFn, and then incubated with serially diluted rMSNOX protein (1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 μg/well). Wells coated with 1 μg of BSA were used as the negative control.

Supplementary Files

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