

1 Forsythoside A blocks the infection of Avian Infectious 2 Bronchitis Virus through binding S1 subunit

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17 **Abstract**

18 Avian infectious bronchitis (IB) is an acute highly contagious respiratory infectious disease
19 caused by avian infectious bronchitis virus (IBV), causing serious economic losses to poultry
20 industry. Forsythiaside A (FTA) is a phenylethanol glycoside compound in the fruit of *Forsythia*
21 *suspensa*, which is widely used for inflammatory respiratory tract disease, including viral infected
22 inflammation. In this study, the mechanism of FTA against IBV was researched. To determine the
23 inhibitory effect of FTA on IBV infection and the interaction between FTA and IBV particles,
24 immunofluorescence assay, plaque assay, RT-qPCR and enzyme-linked immunosorbent assay
25 (ELISA) were performed and it was discovered that FTA inhibited IBV infection and viral
26 attachment to host receptor under non-cytotoxic concentrations, and FTA may bind to virus
27 particles. Then ELISA and isothermal titration calorimetry (ITC) were employed to further study
28 the interaction between FTA and S protein. The results indicated that FTA bound to S1 subunit
29 rather S2 subunit. Besides, the molecular docking showed that FTA form hydrogen bonds with
30 eight amino acids in the S1 subunit. When these amino acids were mutated, the affinity
31 disappeared between the S1 mutant and FTA. In conclusion, FTA prevents IBV infection by
32 blocking viral attachment and contacts with the S1, but not the S2 subunit of IBV. At last, the bind
33 sites were identified by comparing the affinity of wild S1 and mutant S1.

34 **Key words:** Forsythoside A; Avian Infectious Bronchitis Virus; Spike protein; S1 subunit.

35

1. Introduction

Avian infectious bronchitis (IB) is an acute highly contagious respiratory infectious disease caused by avian infectious bronchitis virus (IBV)^[1]. IB not only lowers egg production and quality but also has a negative impact on the worldwide economy, making it a serious issue for the poultry industry^[2]. The most common symptoms of IB are related to the respiratory tract, including wheezing, coughing, sneezing, trachea rale and runny nose^[3]. Although the main target of IBV is the epithelial surface of the respiratory tract, it can replicate in other organs, including the kidney, gastrointestinal tract, urinary tract and reproductive tract, depending on the virus strain^[4].

At present, vaccination is still the most popular way to prevent production loss. However, due to the poor cross protection to mutable and different serotype IBV strains, the current vaccines are insufficient to provide adequate protection^[5]. Forsythiaside A (FTA) is the main active ingredient isolated from *Forsythia suspensa*, which has significant biological activity, showed notable pharmacological properties in inflammatory disease, virus infection, neurodegeneration, oxidative stress, liver injury, and bacterial infection^[6]. In 2019, coronavirus disease 2019 (COVID-19) pneumonia began widely prevalent by SARS-CoV-2. FTA as a part of Lianhuaqingwen capsule, a herb medicine product, was found that angiotensin-converting enzyme 2 (ACE2), the receptor of SARS-CoV-2, could be inhibited by the component. Besides, the molecular docking result showed that FTA could bind to the active region of ACE2 which interact with viral S protein (Asp350 to Arg393)^[7]. The main protease (Mpro) of SARS-CoV-2 is an important non-structural protein mediates viral replication and transcription. It was found that the Mpro enzymatic activity was inhibited at IC₅₀=22.26 μM of FTA and the docking results indicated that FTA was able to bind some sites of Mpro^[8]. What's more, FTA also had a direct virucidal effect on IBV^[9]. But at the detailed anti-IBV role and the

58 potential target remain unknown.

59

2. Materials and Methods

2.1. Cells, viruses, and reagents

African green monkey kidney cells (Vero) and Human Embryonic Kidney 293T (HEK293T) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS, HyClone, USA) containing penicillin (100 units/ml) and streptomycin (100 µg/ml) (Gibco, USA). All cells were grown at 37 °C containing 5% CO₂ in incubator.

The IBV strain Beaudette (GenBank accession number: MZ368698.1), which is maintained in our laboratory, was used for all experiments and is represented as “IBV” in this article.

Forsythiaside A (FTA), purity ≥ 98%, was used for the in this study (Desite, China).

2.2. Virus titration

Vero cells grown in 96-well plates were infected with ten-fold serial dilutions of IBV. After 1 h at 37 °C, the culture medium was replaced with fresh DMEM-2% FBS. Viral titers were determined using endpoint dilution analysis at 5 days post-inoculation. The Reed–Muench method was used to calculate the 50% tissue culture infected dose (TCID₅₀).

2.3. Cytotoxicity assay

The cytotoxic effects of FTA on Vero cells were measured by MTT assay. Briefly, Vero cells were seeded and grown to 90% confluence in 96-well cell culture plates (Corning Incorporated, USA), the supernatant was discarded and the cells were washed with Phosphate Buffered Saline (PBS, HyClone, USA) three times. Different concentrations of FTA were incubated with Vero cells at 37 °C for 48 h. Then the medium was discarded, 100 µL of MTT (1 mg/mL) (Solarbio, China) was added in each well and maintained at 37 °C for 4 h. The formed formazan crystals were dissolved in 150 µL dimethyl sulfoxide (DMSO) (Solarbio, China). The OD value was

82 measured with a microplate reader at 490 nm. The percentage of surviving cells were calculated
83 and used the following formula: cell viability (%) = (OD_{490nm} drug) / (OD_{490nm} control) × 100%.
84 The highest nontoxic concentration of FTA was chosen as the most safety concentration. The
85 experiments were performed in triplicate.

86 2.4. Quantitative reverse transcription PCR (RT-qPCR)

87 Total RNA was extracted from IBV-infected cells at 8 hour-post-infection (h.p.i.), using
88 FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme, China). Subsequently, Evo M-MLV RT
89 Premix for qPCR (Agbio, China) was used to synthesize cDNA and quantification of viral gene
90 expression was performed on the Mx3005P Real-Time PCR System (Stratagene, USA), using
91 Ultra SYBR Mixture (Low ROX) (CWBIO, China). The genomic of IBV Beaudette Nucleocapsid
92 was detected by the following primers: forward: 5'- GAACAGGACCAGCCGCTAA -3' and
93 reverse 5'- GAGGAATGAAATCCCAACG -3'. β-actin gene was used as a reference gene and
94 detected using the primers: forward: 5'- AGGCATCCTCACCTGAAGTACC -3' and reverse 5'-
95 CCACACGCAGCTCATTGTAGAAGG -3'. The qPCR reaction procedure as follows: 10 min at
96 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C, then 15 s at 95 °C, 60 s at 60 °C and 15 s at
97 95 °C. Each sample was performed in triplicate. The relative quantitation of IBV-N gene
98 expression was determined by comparison to β-actin.

99 2.5. Virus pre-treatment assay

100 To observe the effect of FTA treatment at different times, plaque assay and RT-qPCR were
101 performed. With the intent to assess the preventive effect of FTA, Vero cells were seeded in 6-well
102 plates and treated with concentration of 50 μM, 100 μM or 200 μM of FTA for 12 h at 37 °C
103 respectively, then the drug was abandoned, and cells were washed three times with PBS.

104 100TCID₅₀ of IBV was incubated with Vero at 37 °C for 1 h, then removed the virus and washed
105 three times. For plaque assay, cells were overlaid with DMEM containing 2% methylcellulose
106 (Solarbio, China) and 2% FBS and incubated for 48 h at 37 °C. The cells were overlaid with 1%
107 crystal violet (Solarbio, China) and incubated for an additional 30 min at room temperature.
108 Plaque-forming units were counted. The viral infection rate was calculated as the formula: Viral
109 infection rate (%) = (the number of plaques of FTA treated)/ (the number of plaques of IBV
110 infected) × 100%. DMEM treatment was included as control in each condition. All the
111 experiments were performed in triplicate.

112 2.6. Virus attachment assay

113 With a view of study whether FTA could inhibit attachment of IBV, Vero cells were
114 prechilled at 4 °C for 1 h and subsequently challenged with 100TCID₅₀ of IBV in the presence of
115 FTA for 1 h at 4 °C. After washed three times with ice-cold PBS, the cells were coated with 2%
116 DMEM at 37 °C for 48 h. Viral plaque assay was performed. The viral infection rate was
117 calculated as the formula: Viral infection rate (%) = (the number of plaques of FTA treated)/ (the
118 number of plaques of IBV infected) × 100%. DMEM treatment was included as control in each
119 condition. All the experiments were performed in triplicate.

120 2.7. Virus entry assay

121 To evaluate the effect of FTA on the entry of IBV, Vero cells were prechilled at 4°C for 1 h
122 before inoculation with 100TCID₅₀ of IBV for 1 h at 4 °C. The cells were then treated with FTA
123 and further incubated at 37 °C for 1 h. At the end of the incubation, extracellular virus was
124 inactivated by citrate buffer (pH 3.0) for 1 min and then washed with PBS three times before
125 overlaying with 2% DMEM for 48 h at 37 °C. For plaque assay, The viral infection rate was

calculated as the formula: Viral infection rate (%) = (the number of plaques of FTA treated)/ (the number of plaques of IBV infected) × 100%. DMEM treatment was included as control in each condition. All the experiments were performed in triplicate.

2.8. Virus post-infection assay

To investigate the effect of FTA after viral entry, Vero cells were infected with 100TCID₅₀ of IBV for 1h at 37°C, then washed before overlaid medium containing 50 μM, 100 μM, 200 μM of FTA. Viral plaque assay was performed. The viral infection rate was calculated as the formula: Viral infection rate (%) = (the number of plaques of FTA treated)/ (the number of plaques of IBV infected) × 100%. DMEM treatment was included as control in each condition. All the experiments were performed in triplicate.

2.9. Viral inactivation assay

100TCID₅₀ of IBV was mixed with 50 μL of 50 μM, 100 μM, 200 μM of FTA respectively and incubated at 37 °C for 1 h. The mixture was then diluted 20-fold with no phenol red DMEM, and the dilution was subsequently added to the monolayers of Vero cells which were seeded in 6-well plates. After adsorption for 1 h at 37 °C, the medium was discarded, and the cells were washed with PBS three times. Then the cells were overlaid with the maintenance medium and further incubated at 37 °C for 48 h. Viral plaques were counted as previously described. The experiments were performed in triplicate.

2.10. Immunofluorescence assay (IFA)

Vero cells were seeded in 12-well plates including 20 mm coverslips (Solarbio, China) and pre-treated with 50 μM, 100 μM or 200 μM of FTA, respectively, for 1 h at 37 °C and then infected with IBV for 1 h at 37 °C. Cells were washed with PBS and then incubated in fresh

148 medium containing FTA for 48 h. DMEM served as the treatment control. Subsequently, the
149 overlay was removed, and the coverslips were rinsed three times with PBS. The cells were then
150 fixed with 4% PFA (Solarbio, China) for 15 min at room temperature (RT) and blocked with
151 QuickBlock™ Blocking Buffer (Beyotime, China) for 1 h. The anti-IBV-chicken positive serum
152 (B0116, QYH Biotech, China) was used as primary antibody (1:500) and incubated at 4°C
153 overnight. Rabbit anti-chicken IgG conjugated with Cy3 (K1035R-Cy3, Solarbio, China) was used
154 as the secondary antibody (1:1000) and incubated at room temperature for 1 h in the dark. Hoechst
155 33342 (C1022, Beyotime, China) was used to stain the cell nuclei for 5 min at room temperature.
156 Coverslips were mounted on a microscope slide and viewed with Leica Confocal Microscopes
157 Stellaris 5. Representative images were obtained and processed using the LAS X Navigator
158 software.

159 2.11. Alignment of FTA and IBV S protein

160 The 3D structural diagram of IBV S protein (6CV0) was taken from Protein Data Bank
161 (<http://www.rcsb.org/pdb/>), and the 3D structure diagram of FTA was drawn by Chem3D software.
162 Autodock was used to calculate and the docking result was showed by PyMOL.

163 2.12. Plasmid construction

164 Genes encoding IBV-Beaudette-S1 (the aa 1-532 of precursor of S protein) and
165 IBV-Beaudette-S2 (the aa 539-1091 of precursor of S protein) were amplified from Vero cells
166 infected with IBV-Beaudette by reverse-transcription PCR (RT-PCR). The amplified fragment was
167 ligated into the pCDNA3.4 vector (Addgene, USA) with a C-terminal 6×His-tag tag. The
168 IBV-Beaudette-S1 mutant [(aa 434, 440, 441, 454, 469, 471, 473, 485 were mutated to

169 phenylalanine (aa 434), alanine (aa 440), alanine (aa 441), valine (aa 454, aa 469 and aa 471),
170 asparagine (aa 473) and phenylalanine (aa 485)] was synthesized by Detai Bioscience, Inc.

171 *2.13. Expression and purification of recombinant proteins*

172 The pCDNA3.4 expression vectors containing the described sequences above was transfected
173 into HEK293T cells by the measure of GP-transfect-Mate (GenePharma, China) for 48 h at 37 °C.
174 Then the medium was discarded, and the cell lysate was harvested. The lysate of HEK293T with
175 no plasmid was regarded as control. The proteins were purified by the manual of Ni-IDA agarose
176 gel (Detai Bioscience, China), and the products were stored at -80 °C.

177 *2.14. SDS-PAGE and Western blotting*

178 The expression and purification of the proteins were analyzed by Western blotting and
179 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The cell lysate was
180 mixed with 5 × SDS-PAGE Sample Loading Buffer (Beyotime, China) and boiled at 100 °C for
181 10 min. Equal amounts of protein were separated on a 10% SDS polyacrylamide gel and
182 electro-transferred from the gel to a nitrocellulose filter (NC) membrane. After blocking with 5%
183 skimmed milk powder in Tris-HCl buffer with Tween-20 (TBST) (LABLEAD, China), the
184 membrane was incubated with Rabbit His-Tag antibody (1:1000) (#12698, Cell Signaling
185 Technology, USA) at 4 °C overnight and IRDye® 800CW Goat anti-Rabbit IgG (1:15000)
186 (926-32211, LI-COR, USA) at room temperature for 1 h. β-actin was used as the internal loading
187 control. The membrane was scanned by Odyssey Infrared Imaging (LI-COR, USA). The purified
188 protein was mixed with Loading Buffer and boiled. 10% SDS polyacrylamide gel was used to
189 separate the protein and then stained by Coomassie blue solution for 30 min and the gel was
190 decolorized by ddH₂O overnight.

191 2.15. The conjugation of FTA and OVA

192 FTA (0.04 mmol), succinic anhydride (0.04 mmol) (Leyan, China) and triethylamine (0.04
193 mmol) (Sinopharm, China) were dissolved in N, N-Dimethylformamide (DMF) (500 μ L)
194 (Macklin, China) and pyridine (500 μ L) (Macklin, China). The mixture was stirred at room
195 temperature for 2 h, then the mixture was evaporated by vacuum drying oven at room temperature
196 and the crystals were collected.

197 The crystals were completely dissolved in 500 μ L DMF, then 0.2 mmol
198 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Sigma, USA) and 0.2
199 mmol N-Hydroxy succinimide (NHS) (Macklin, China) were added while stirring, and the mixture
200 was stirred at room temperature overnight. The supernatant was collected after centrifugation
201 which was regarded as solution A. 15 mg ovalbumin (OVA) (Macklin, China) was dissolved in 5
202 mL PBS which was regarded as solution B. Under magnetic stirring, solution A was added drop by
203 drop to solution B and mixed at room temperature for 12 h. When the reaction finished, the
204 supernatant was collected and dialyzed at 4 $^{\circ}$ C for 3 d by dialysis bag (14000 kD, Beyotime,
205 China), changed dialysate (PBS) three times a day. The solution in the dialysis bag was obtained
206 and the precipitate was discarded. To determine whether the conjugation of FTA and OVA was
207 successful, the 190 nm - 400 nm spectrum of the mixture was scanned by DeNovix DS-11
208 Spectrophotometer/Fluorometer Series.

209 2.16. ELISA

210 FTA-OVA in carbonate buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.6) were coated in 96
211 well flat bottom polystyrene plates and incubated at 4 $^{\circ}$ C overnight. Then the plates were washed
212 three times with 200 μ L Phosphate Buffered Saline with Tween-20 (PBST) and blocked for 1 h at

213 room temperature with 100 μ L 5% skimmed milk powder in PBS. After that the plates were
214 washed three times with PBST and two-fold diluted IBV, IBV-Beaudette-S1 or IBV-Beaudette-S2
215 were added in plate and incubated for 1 h at 37 °C. PBS or HEK293T lysis were used as negative
216 control. Followed three times washed with PBST, primary antibody [Infectious Bronchitis virus
217 Nucleoprotein antibody (1:1000) (B819M, GeneTex, USA) or Rabbit His-Tag antibody (1:1000)
218 (#12698, Cell Signaling Technology, USA)] and secondary antibody [Goat anti-mouse IgG
219 conjugated with HRP (1:1000) (SE131, Solarbio, China) or Goat anti-rabbit IgG conjugated with
220 HRP (1:1000) (SE134, Solarbio, China)] were incubated for 1 h at 37 °C, respectively. 100 μ L
221 TMB (Solarbio, China) was added in each well at room temperature for 60 min and the reaction
222 was terminated by Stop Solution for TMB Substrate (Solarbio, China). The absorbance was
223 immediately determined at 450 nm. The OD_{450nm} was calculated and the P/N=2.5-3.0 was
224 regarded as positive.

225 2.17. Isothermal titration calorimetry (ITC)

226 FTA was prepared as 1 mM in PBS and loaded in sampling needle. S1 protein (1 μ M) was
227 dissolved in PBS then added to the sample cell. The titration reaction temperature was 25 °C, the
228 number of drops was set to 20, reference power was 10 μ cal/s, single titration volume was 2 μ L,
229 the single injection time of FTA was 4 s, the interval time between two titrations was 180 s, and
230 the agitator speed was 1000 rpm/min. When titrating S2 protein, the concentration of S2 protein
231 was 1 μ M. FTA concentration was changed into 100 μ M, the titration interval was set to 60 s, and
232 other conditions remain unchanged.

233 2.18. Statistical analysis

234 All data were expressed as the mean $\pm$ standard error of the mean (SEM) from three
 235 independent experiments performed in triplicate. The statistical analyses were conducted using
 236 Student's t-test in GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). A
 237 p-value was considered significant as the following: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p <$
 238 0.0001)
 239

240 **3. Results**

241 *3.1. The cytotoxic effect of FTA on Vero cells*

242 To investigate whether FTA treatment affects cell viability, the toxicity of FTA on Vero cells
243 was determined by MTT assay. As the data shown, the cell viability was slightly decreased at 300
244 μM , and the viability was decreased approximately 20% and 40% at the concentration of 400 μM
245 and 500 μM , respectively (Fig.1). These results indicated that FTA did not influence cell viability
246 at concentrations below 300 μM . Therefore, the concentration of 50 μM , 100 μM and 200 μM of
247 FTA were chosen for the subsequent antiviral assays.

248 *3.2. FTA inhibits IBV infection*

249 Immunofluorescence assay (IFA) was performed to study whether FTA could inhibit IBV
250 infection. As the result shown, when the concentration of FTA was increased, the red
251 fluorescence, high indicated as IBV particles, was faded (Fig.2), indicating that FTA has an effect
252 of anti-IBV infection, but the mechanism not revealed.

253 *3.3. FTA inhibits the attachment of IBV and may bind to IBV particles*

254 To better understand the antiviral mechanism and the stage of IBV infection affected by FTA
255 *in vitro*, plaque assay and RT-qPCR were performed. As the concentration of FTA increased, the
256 plaque formation of stage of viral attachment decreased. However, the plaque formation of the
257 stages of FTA pretreatment, viral entry and viral post-infection were not decreased by FTA
258 treatment (Fig.3). After plaques were counted and RT-qPCR was performed, the results showed
259 that there was no inhibition effect of FTA on IBV entry (Fig.4C, Fig.4H), not prevent IBV
260 infection either (Fig.4A, Fig.4F) and have no effect in the post-infection stage. (Fig.4D, Fig.4I).
261 But viral attachment was decreased by FTA treatment (Fig.4B, Fig.4G).

262 The adsorption of IBV requires interaction between S protein and receptors (alpha2,3-linked
263 sialic acids) on the host cell^[10]. However, the pretreatment by FTA for 12 h didn't reduce the
264 infection of IBV on Vero cells. This result indicated that there was not an interaction between FTA
265 and cell, so we assumed that the efficacy of FTA can only be achieved by combining with IBV
266 particles. To verify this hypothesis, different concentrations of FTA were mixed with 100 TCID₅₀
267 IBV at 37 °C for 1 h, then the mixtures were diluted 20-fold to avoid the effect to cells. The plaque
268 assay result showed that viral infection rate decreased when the concentration of FTA increased

269 (Fig.3, Fig.4E). The results indicated that the inactivation of IBV infection by FTA is characterized
270 by reduced attachment, and FTA may bind to IBV particles.

271 3.4. Synthesis of artificial antigen FTA-OVA

272 Hapten refers to a low molecular weight compound that cannot trigger the immunogenic
273 reaction of producing antibodies and should be covalently coupled with immunogenic
274 macromolecules (carrier protein) to obtain immunogenicity. According to this, a carboxyl needs to
275 be introduced into FTA using the succinic anhydride method, to further synthesize artificial
276 antigens. After the hapten was contributed and conjugated with OVA, FTA-OVA was determined
277 by a spectrophotometer. Red indicated the spectrum of OVA, with a characteristic absorption peak
278 at approximately 228 nm; Blue represented the spectrum of FTA, with a characteristic absorption
279 peak at approximately 327 nm; Green was the spectrum of the synthesized artificial antigen. It has
280 two absorption peaks at about 203 nm and 349 nm, including the characteristic absorption peaks
281 of OVA and FTA, indicated that the artificial antigen was successfully synthesized (Fig.5).

282 3.5. Expression and purification of IBV S1 protein, S2 protein

HEK293T cells were transfected with plasmid encoding S1 protein or S2 protein, after 48 h incubation, the protein was collected and purified. The result of western blotting showed that there were approximately 100 kDa and 90 kDa bands indicated S1 protein and S2 protein, respectively (Fig.6A, Fig.6B). SDS-PAGE showed the result of purification. The lanes 1, 2 represented unpurified lysis solution and flow-through liquid, respectively (supplementary in Fig.S1A, Fig.S1B). In purification of S1 protein or S2 protein, lanes 3-4 (supplementary in Fig.S1A) or lanes 3-8 (supplementary in Fig.S1B) were collected after 100 mM imidazole elution, lanes 5-7 (supplementary in Fig.S1A) or lanes 9-12 (supplementary in Fig.S1B) were collected after 500 mM imidazole elution, S1 protein or S2 protein were mainly present in the eluent of lane 6 (supplementary in Fig.S1A) or lane 9 (supplementary in Fig.S1B).

3.6. FTA bound to IBV S1 subunit but not S2 subunit

After the conjugation of FTA and OVA, FTA was used as a coating antigen for ELISA, then 800TCID₅₀ IBV was added and ratio diluted 2-fold. DMEM contain 2% FBS was set as the control. The result of ELISA showed that the ratio of OD_{450nm} of positive/negative (P/N) increased following the amount of IBV added, revealed that FTA could indeed interact with IBV particles (Fig.7A). When the purified S1 protein was added in same method, the ratio of OD_{450nm} of P/N also increased (Fig.7B), preliminary identified that S1 subunit was bound to FTA.

ITC was performed to further determine the interaction between FTA and S1 subunit. In the upper part of figure 8A, as the amount of FTA titration increased, the absorption heat of S1 and FTA reactions decreased, indicated that the binding between FTA and S1 protein tends to be saturated. In the lower part of figure 8A, the fitting curve was close to the "S" shape, and it was found that as the number of moles of FTA titration increased, the reaction between the two

substances had a maximum rate, indicated that FTA interacted with the S1 subunit of IBV (Fig.8A).

There is a report found that IBV S2 subunit has a certain promoting effect on viral adsorption^[11]. ELISA and ITC were performed and the results excluded the influence of S2 subunit on IBV attachment. The result of ELISA showed there were no significant changes of the ratio of OD_{450nm} of P/N when added S2 protein (data not shown), and when S2 protein was titrated with FTA, no regular peak was observed, almost no heat change, and the scatter diagram was unordered (Fig.8B), suggested FTA did not bind to IBV S2 subunit.

3.7. Alignment of FTA and IBV S protein

Using Autodock software, FTA might form hydrogen bonds with serine (aa 454) and tyrosine (aa 485) of IBV S protein (Fig.9A), and tyrosine (aa 434), threonine (aa 440), glycine (aa 441), serine (aa 469 and aa 471) aspartate (aa 473) interacted with FTA (Fig.9B). The donors and acceptors of hydrogen bond between FTA and IBV S protein were showed (Fig.9C). These amino acids all locate at S1 subunit of IBV S protein, and S1 subunit mediates IBV attachment, corresponded to the results of plaque assay and RT-qPCR previously.

3.8. IBV Beaudette S1 mutant loss binding ability with FTA

To explore the functions of these sites on FTA binding, S1 mutant was generated by mutating the eight amino acids into phenylalanine (aa 434), alanine (aa 440), alanine (aa 441), valine (aa 454, aa 469 and aa 471), asparagine (aa 473) and phenylalanine (aa 485) (supplementary in Table S1). Firstly, the expression of S1 mutant was detected by western blotting. Corresponded to the S1 protein, S1 mutant was approximately 100 kDa (Fig.10A, Fig.10B). Then the interaction between S1 mutant and FTA was explored by ELISA and ITC. When the molar concentration of S1 protein

and S1 mutant was close, the ratio of OD_{450nm} of P/N of S1 mutant was decreased. What's more, the result of ITC showed that there was almost no heat change when FTA and S1 mutant titration (Fig.10C, Fig.10D), indicated that the affinity disappeared after mutated those sites, revealed that FTA could bind these sites, but not found which one was FTA exactly bound.

4. Discussion

Coronavirus infect cells can be roughly divided into the following steps: attachment, entry, replication, assembly, and release^[12]. To explore the anti-viral mechanism of FTA, plaque assay and RT-qPCR were performed to test the effect of FTA on each step of IBV infection. It was found that FTA inhibited IBV attachment to surface of Vero cells membrane. Pretreated cells with FTA, IBV infection couldn't be prevented; but once IB virus incubated with FTA, the infection rate decreased. These results indicate that the inhibition of IBV attachment by FTA is due to interaction with viral particles, rather than with cellular receptors. Spike protein is the largest structural protein of coronavirus, which forms rod like protrusions on the surface of viral envelope and exists in the form of dimer or trimer^[4]. The S protein of IBV is a highly glycosylated Class I fusion protein^[13]. It has a special pentapeptide motif RRFRR on its amino acid sequence, which can be recognized and cleaved by the furin-like protease in the host cell. Subsequently, the S protein is divided into S1 subunit containing approximately 500 amino acids and S2 subunit containing approximately 600 amino acids^[10, 14]. S1 subunit is an important part for IBV to exert adsorption function, and all known receptor binding domains (RBDs) are located on the S1 subunit^[15]. According to the results above, S1 subunit might be the binding part of FTA. The docking results showed that there were eight sites where FTA probably bind to S protein, and the sites were all located at S1 subunit. S2 subunit contains an extracellular domain, a transmembrane

domain, and an intramembrane domain, which are anchored to the viral envelope through the intramembrane domain to mediate the fusion of the viral envelope and cell membrane^[16]. What's more, S2 subunit could promote virus attachment^[11]. Then S1 and S2 protein were expressed and purified, and molecular interaction assays were performed. Both ELISA and ITC results indicated that FTA interact with S1 subunit rather than S2 subunit. In order to further confirm that FTA bound to the eight amino acids, we chose other amino acids with similar structures to replace the amino acids of wild S1 subunit. As table S1 showed, the R group of amino acids of S1 mutant were different from amino acids of S1 wild, and these mutated amino acids might cause inability to generate hydrogen bonds or weakening of bond energy with FTA. Such as tyrosine, which was converted to phenylalanine, lost phenolic hydroxyl groups, might interfere with the formation of hydrogen bonds, might result in S1 mutant loss affinity to FTA, but which amino acid plays a decisive role is still unknown.

When these sites were mutated into another amino acids with similar property, the affinity of mutant to FTA was lower than S1 protein (in similar weight), so we considered that FTA might block the interaction between S protein and certain receptors, rather than known receptors. The effect of each site which we found in this study should be explored in the future.

5. Conclusion

The molecular mechanism of anti-IBV infection and the targets of FTA were identified. FTA prevents IBV infection by blocking viral attachment and contacts with the S1 subunit of IBV, but not with the S2. At last, the bind sites were identified by comparing the affinity of wild S1 and S1 mutant.

372 **Declarations**

373 **Ethics approval**

374 Ethical approval is not required.

375 **Competing interests**

376 The authors declare that they have no competing interests and approved the manuscript,

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422

423 **Figure captions**

424 **Fig.1 FTA had cytotoxicity on Vero cells.** Vero cells were treated with different concentrations of
425 FTA and cell viability was detected by MTT assay. The experiment was repeated three times. $*p <$
426 0.05 , $****p < 0.0001$, compared with $0 \mu\text{M}$.

427 **Fig.2 Identification of anti-IBV activity of FTA in Vero cells.** Vero cells were pre-treated with
428 the indicated concentrations of FTA for 1 h and then infected with IBV for 1 h at 37°C . Cells were
429 washed with PBS and then incubated in fresh medium containing FTA for 48 h. DMEM served as
430 the treatment control. At 48 h.p.i., IFA images of Vero IBV-infected and FTA-treated cells were
431 collected. Cell nuclei were indicated by Hoechst33342 for blue, IBV particles were indicated by
432 Cy3 for red.

433 **Fig.3 The effect of FTA addition time on the plaque formations.** Plaque formations of plaque
434 assay of each stage. The experiment was repeated three times.

435 **Fig.4 The effect of FTA on the different stages of IBV infection.** Plaque assay and RT-qPCR
436 were used to determine the stage of IBV infection. (A, F) FTA pre-treatment assay. (B, G) Viral
437 attachment assay. (C, H) Viral entry assay. (D, I) Viral post-infection assay. (E) Viral inactivation
438 assay. The experiment was repeated three times. $*p < 0.05$, $**p < 0.01$, compared with $0 \mu\text{M}$.

439 **Fig.5 The artificial antigen FTA-OVA has been synthesized.** Succinic anhydride method and
440 carbodiimide method were used to synthesize FTA-OVA. The spectrum of three substances. Red
441 indicated the spectrum of OVA, blue represented the spectrum of FTA and green was the spectrum
442 of FTA-OVA.

443 **Fig.6 The expression and purification of S1 protein and S2 protein.** (A) Western blotting
444 showed that there was a band at approximately 100 kDa , indicated S1 protein. (B) Western

445 blotting showed that there was a band at approximately 90 kDa, indicated S2 protein.

446 **Fig.7 FTA interacted with IBV particles and S1 protein.** FTA-OVA was coated on the ELISA
447 plate as a determined concentration, then added (A) virus or (B) S1 protein with twice dilution
448 ratios, the ratio of OD_{450nm} of positive/negative increased following the amount of virus or protein
449 added. (A) The DMEM culture medium contain 2% FBS or (B) PBS was set as the control.

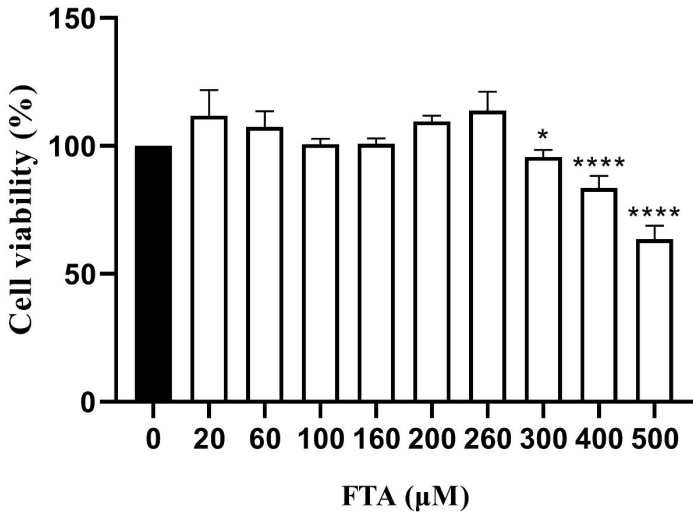
450 **Fig.8 The results of FTA titrated S1 protein or S2 protein.** (A) S1 protein was titrated by FTA.
451 In the upper figure, the heat absorbed was decreased with adding FTA. In the lower figure, the
452 fitting curve was close to the "S" shape, and there was a max gradient. (B) S2 protein was titrated
453 by FTA. There is no observed regular peak, almost no heat change, and the scatter diagram was
454 unordered.

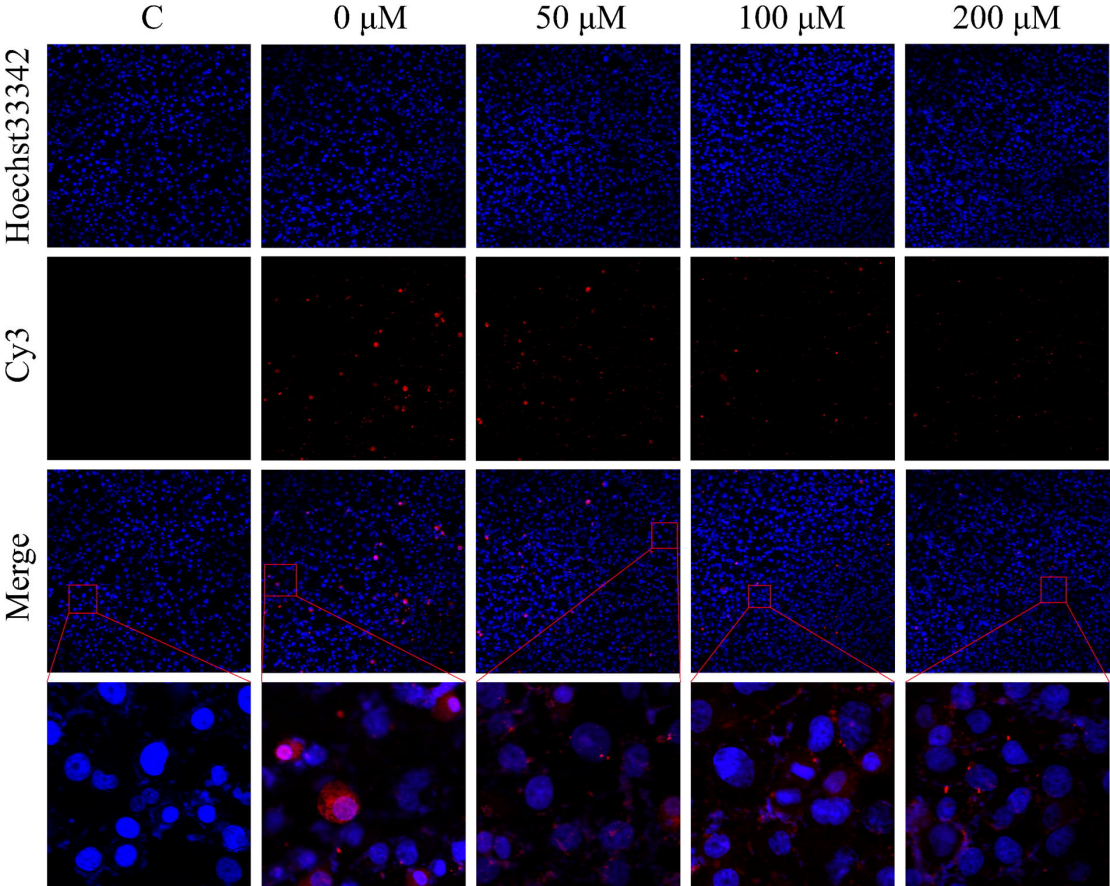
455 **Fig.9 Docking results of FTA and IBV S protein.** (A) FTA bound to serine (aa 454) and tyrosine
456 (aa 485) of S protein by hydrogen bond (red dashed line). (B) Docking results showed that
457 tyrosine (aa 434), threonine (aa 440), glycine (aa 441), serine (aa 469 and aa 471) and aspartate
458 (aa 473) interacted with FTA. Dark green line indicated that FTA interacted with amino acids by
459 conventional hydrogen bond, light green line indicated that FTA interacted with amino acids by
460 carbon hydrogen bond. (C) The result of FTA bound to S protein showed the donors and acceptors
461 of hydrogen bond.

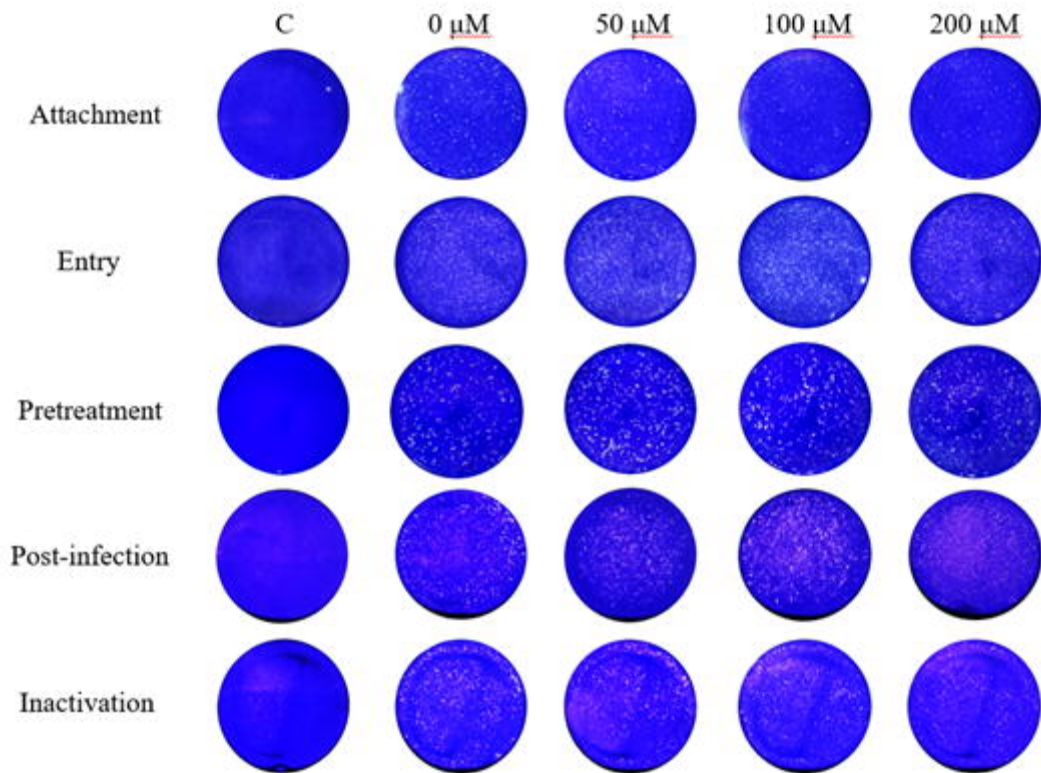
462 **Fig.10 The expression of S1 mutant was showed by western blotting and the comparison of**
463 **affinity of FTA bound to S1 protein or S1 mutant.** (A, B) After the amino acids of S1 protein
464 were changed into another amino acids with similar structure and properties, the molecular weight
465 of S1 mutant and S1 protein was close, approximately 100 kDa. (C) The ratio of OD_{450nm} of P/N
466 was decreased when S1 protein was mutated. ****p < 0.001, compared with S1 protein. (D) S1

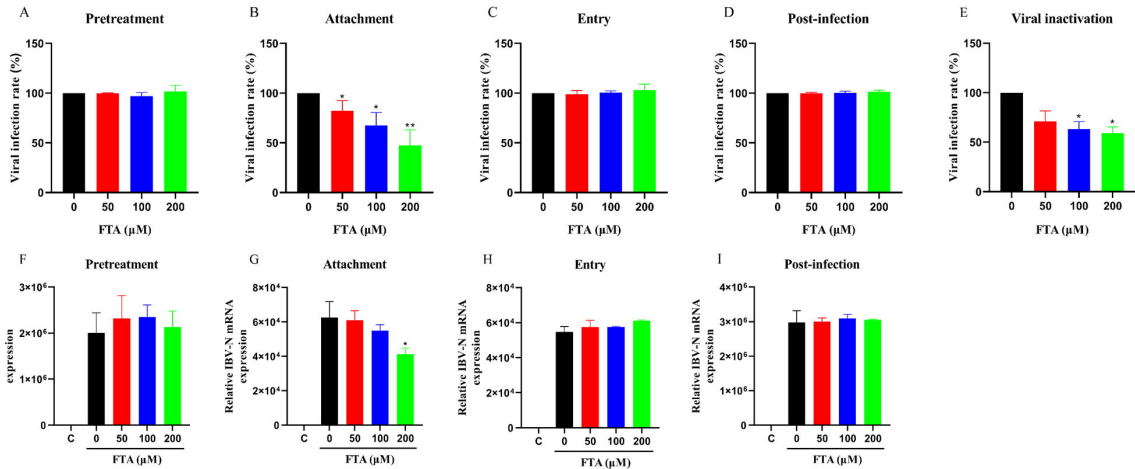
467 mutant was titrated by FTA. Unable to determine whether it is an endothermic or exothermic

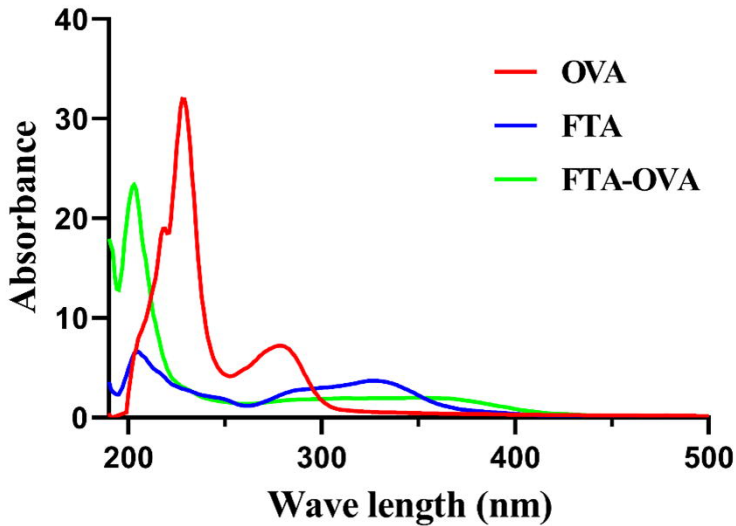
468 reaction, and there was almost no heat change.

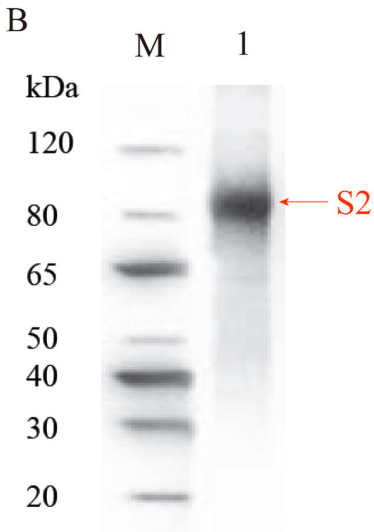
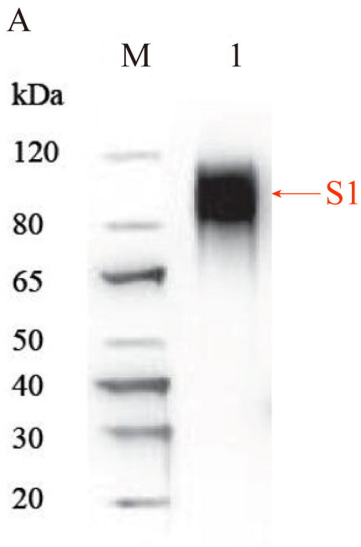




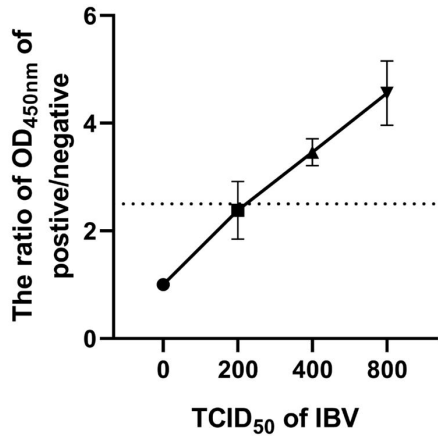




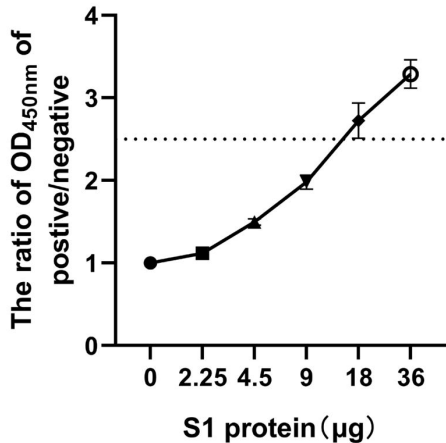


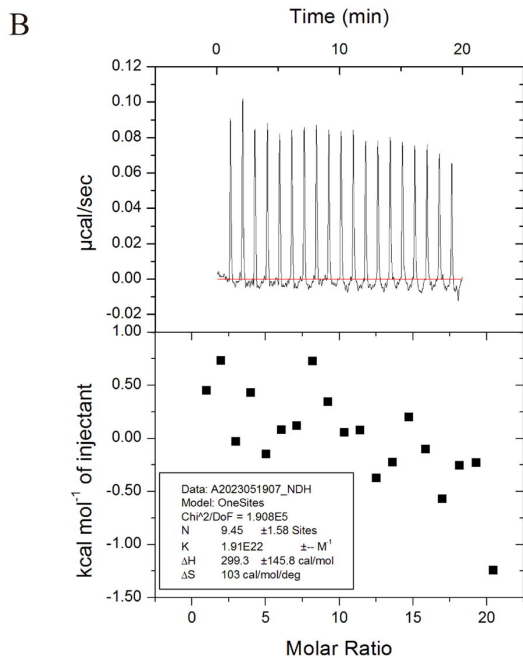
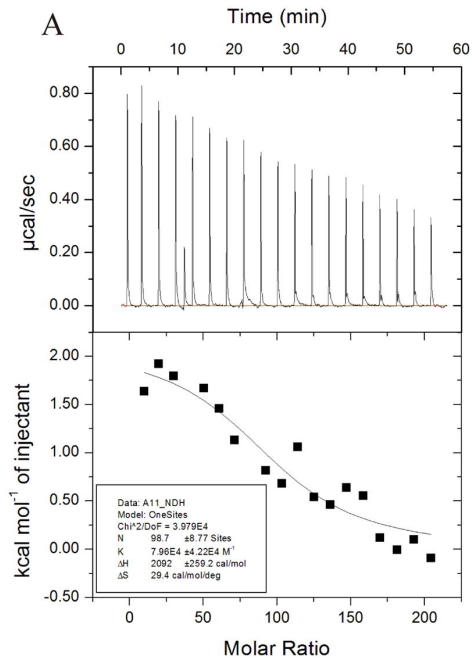


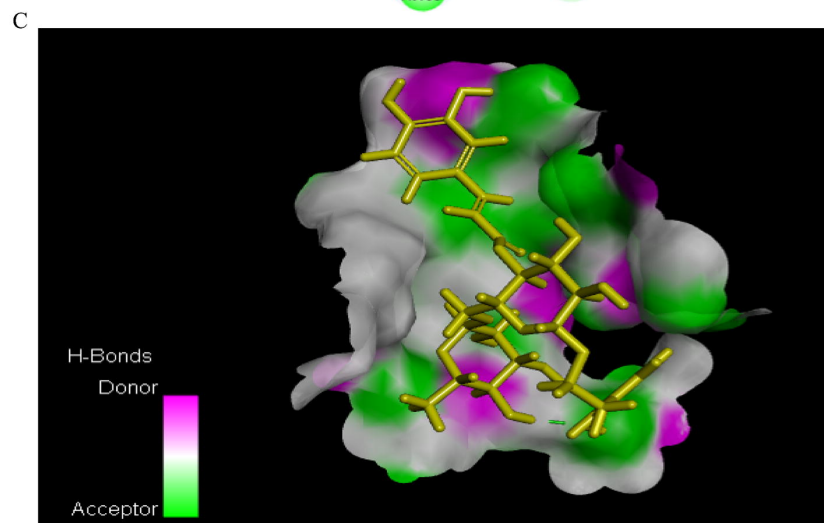
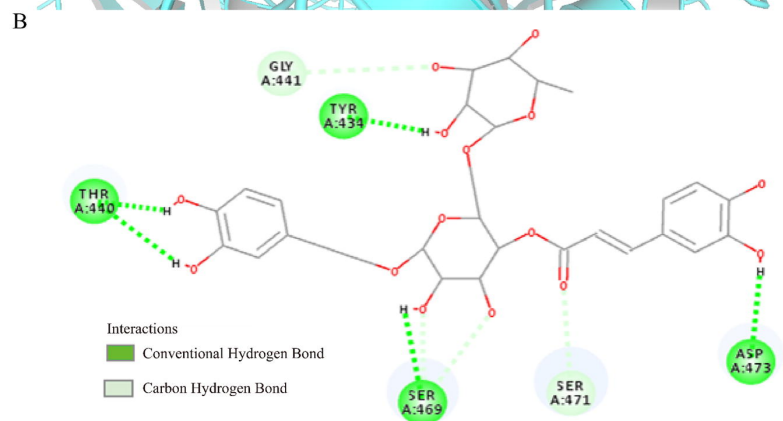
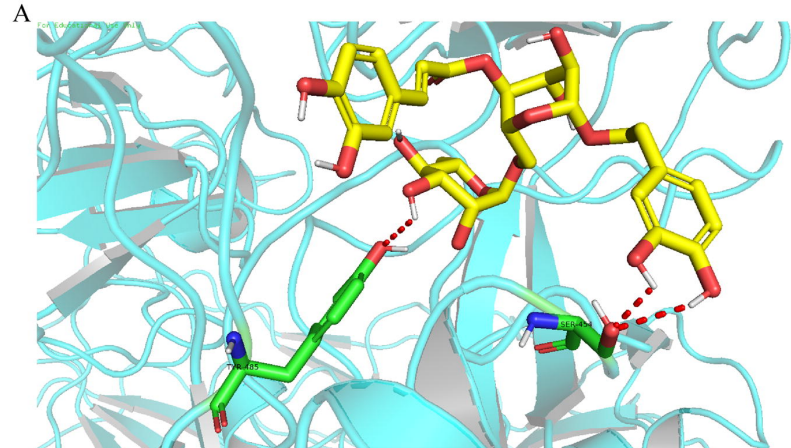
A



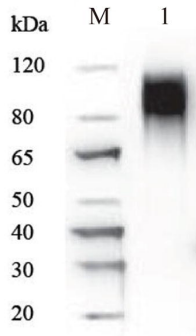
B



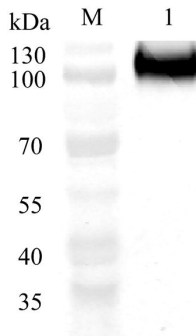




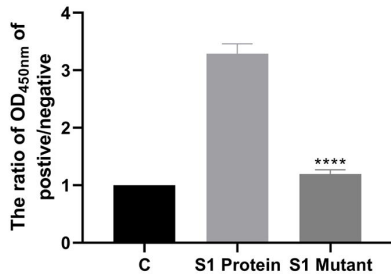
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B



C



D

