

Research Article

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Identificaton of novel *Cystoisospora suis* enolase 2 interaction proteins in IPEC-J2 cells using Co-IP/LC-MS

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Abstract: Enolase 2 (ENO2) is a crucial an important glycolytic enzyme involved in glycolysis and is also recognised as a multi-functional protein. However, its interacting proteins in intestinal porcine epithelial cells (IPEC-J2 cells) remain unclear. This study investigates the interacting proteins of *Cystoisospora suis* (Biester et Murray, 1934) ENO2 (CsENO2) in IPEC-J2 cells. According to our previous research, CsENO2 is highly expressed in sporulated oocysts. Co-immunoprecipitation/liquid chromatography–mass spectrometry (Co-IP/LC-MS) was conducted using the pcDNA3.1-Flag vector carrying the *enolase 2* gene, which was transfected into IPEC-J2 cells to co-immunoprecipitate with cellular proteins. The Co-IP/LC-MS-based protein sequencing identified three cellular proteins: NME2, RPS3, and Vimentin. Subsequently, cDNAs encoding the complete coding sequences of these proteins were cloned into the pGEX-4T-1 expression vector. Immunofluorescence assays revealed the localisation of CsENO2 in sporulated oocysts and sporozoites. Most CsENO2 protein was found in the cytoplasm of sporulated oocysts. In sporozoites, CsENO2 protein was primarily distributed around and outside the nucleus. This study aims to identify CsENO2-interacting proteins in IPEC-J2 cells and determine the localisation of CsENO2 within sporulated oocysts and sporozoites.

Keywords: GST pull-down assay, NME2, RPS3, Vimentin

This article contains supporting table (Table S1) online at <http://folia.paru.cas.cz/suppl/2026-73-009.pdf>

Cystoisospora suis (Biester et Murray, 1934) is an obligate parasitic protist that causes coccidiosis in piglets. It infects the small intestinal epithelial cells of pigs, leading to severe diarrhea and potentially fatal outcomes. In China, affecting approximately 45–100% of pig farms, *C. suis* has a case fatality rate as high as 75%, posing a significant threat to the global pig industry (Gong et al. 2021, Bawm et al. 2022).

Enolase is a key enzyme in glycolysis, catalysing the conversion of glucose to pyruvate while generating ATP and the cofactor NADH, thereby supporting cellular metabolism and vital physiological processes (Xu et al. 2019). Beyond its metabolic role, enolase has been identified as a plasminogen-binding protein in bacteria and parasites (Bernal et al. 2004, Vanegas et al. 2007, Kolberg et al. 2006, Ayón-Núñez et al. 2018, Serek et al. 2020). By binding host plasminogen and promoting its activation in the presence of host tissue plasminogen activator, enolase is

thought to prevent blood clot formation on the pathogen surface (de la Torre-Escudero et al. 2010).

Enolase 2 (ENO2), also known as neuron-specific enolase (NSE) or γ -enolase, is a highly specific marker for neurons and peripheral neuroendocrine cells (Isgrò et al. 2015). Under normal conditions, NSE is restricted to specific tissues; however, elevated levels in body fluids may indicate malignant proliferation, rendering it diagnostically valuable. Recently, NSE has been identified as an excretory/secretory antigen (ESA) in *Toxoplasma gondii* (Nicolle et Manceaux, 1908) (Jiang et al. 2016).

Previous studies have shown that *C. suis* enolase 2 (CsENO2) is highly expressed in sporulated oocysts (Yang et al. 2020). Given that protein-protein interactions may contribute to cell invasion specificity, we conducted a GST pull-down assay to investigate their relevance to CsENO2-dependent cellular functions. This study aims to identify CsENO2-interacting proteins in host mucosal epithelial cells.

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Table 1. Primers used in this study for amplification of the target genes.

Gene	Sequence (5'-3')	Expected size (bp)	Constructions
ENO2-F1	GGGGAATTCATGGAAATCTTCTTCTTTGC (EcoR1)	1482	pcDNA3.1-flag- <i>eno2</i> ; pET32a- <i>eno2</i>
ENO2-R1	TGCTCGAGTTATTTTCGATTACGAAAATCCG (XhoI)		
NME2-F	AAAGAATTCATGGCCACGCGAGCGACCTTC (EcoR1)	459	pGEX-4T-1- <i>nme2</i>
NME2-R	GGGCTCGAGTTATTCATAGATCCAGTCAAAAGC (XhoI)		
RPS3-F	TGGGAATTCATGGCGGTGCAGATTTCCAAG (EcoR1)	732	pGEX-4T-1- <i>rps3</i>
RPS3-R	AAACTCGAGTTATGCTGTGGGTACTGGCTG (XhoI)		
Vim-F	AAGGAATTCATGTCCACCAGGACCGTGTCC (EcoR1)	1284	pGEX-4T-1- <i>vimentin</i>
Vim-R	TGCGGCCGCTCATTGATAACCCCTCAGGTTC (NotI)		
ENO2-F2	GTTTCATATGATGGAAATCTTCTTCTTTGC (NdeI)	1482	pET30a- <i>eno2</i>
ENO2-R2	GCAAGCTTTTATTTTCGATTACGAAAATCCG (HindIII)		

The underlined nucleotides indicate restriction enzyme recognition sites used for cloning.

MATERIAL AND METHODS

Reagents and antibodies

Mouse anti-flag monoclonal antibody (mAb) was purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-GST mAb, mouse anti-His mAb, mouse anti-NME/NM23 nucleoside diphosphate kinase 2 (NME2) mAb, and rabbit anti-ribosomal protein S3 (RPS3) antibodies were purchased from Sangon Biotechnology (Shanghai, China). Protein concentrations were determined by bicinchoninic acid (BCA) assay (Beyotime Biotechnology, Shanghai, China).

Primers, vectors and cell culture

The primers with restriction enzyme sites and vectors are summarised in Table 1. IPEC-J2 cells were cultured in DMEM/Ham’s F-12 medium (Gibco, Thermo Fisher Scientific Inc., Waltham, USA) with 5% foetal calf serum (FCS, Gibco), 100 U/ml of penicillin and 0.1 mg/ml of streptomycin (Gibco).

Collection of *Cystoisospora suis* oocysts

Oocysts of *Cystoisospora suis* (strain LY1) were collected from experimentally infected suckling piglets on the fifth day post-infection, cleaned, sporulated *in vitro*, and stored at 11 °C for later use at the Institute of Fujian Provincial Key Laboratory for the Prevention and Control of Animal Infectious Diseases and Biotechnology, College of Life Sciences, Longyan University, China.

Construction of *enolase 2* gene in pcDNA3.1-flag and pET32a expression vectors

Total RNA was isolated from *C. suis* oocysts using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed to generate full-length cDNAs. Briefly, 2 µg of total RNA was mixed with oligo(dT) primer, incubated at 72 °C for 10 min. RT was carried out at 42 °C for 90 min in 25 µl reaction containing 5 µl of RT buffer, 5 µl of dNTPs (10 mM), RNA template (2 µg), 0.5 µl of reverse transcriptase, and RNase-free water. PCR was subsequently carried out using 50 ng of cDNA, 10 µM forward and reverse primers, 25 µl PrimeSTAR® HS (Premix) (Takara Bio., San Jose, USA) and adjusted with nuclease-free water to a final volume of 50 µl. PCR cycling conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 90 s, and a final extension at 72 °C for 10 min. To construction *enolase 2* into the pcDNA3.1-flag (GE Healthcare, Chicago, USA) and pET32a vectors (Novagen, Madison, WI, USA), the coding sequences (CDSs) were amplified by PCR using ENO2-F1 and

ENO2-R1 (Table 1). The amplified DNA fragments and vectors were digested with the respective restriction enzymes, gel purification, and ligated overnight at 16 °C. The resulting plasmids, pcDNA3.1-flag-*eno2* and pET32a-*eno2*, were transformed into *Escherichia coli* DH5a (Takara Bio.). The accuracy of these constructs was confirmed by PCR and DNA sequencing (Sangon Biotech, Shanghai, China).

Co-immunoprecipitation and liquid chromatography–mass spectrometry (LC-MS) analysis

To identify CsENO2-associated proteins in intestinal porcine epithelial cells (IPEC-J2 cells), cells were transfected with pcDNA3.1-flag (vector control) or pcDNA3.1-flag-*eno2* for 24 h at 37 °C. To obtain sufficient candidate proteins for mass spectrometry analysis, transfected cells from ten 10-cm cell culture dishes were collected and subjected to co-immunoprecipitation using the Catch and Release® v2.0 Reversible Immunoprecipitation System (Merck) according to the manufacturer’s instructions. Briefly, 1,000 µg of total cellular proteins were incubated with 4 µg of Flag monoclonal antibodies overnight at 4 °C. Immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands showing significant differences were excised, washed twice with 50% acetone, and subjected to in-gel trypsin-digestion. The resulting peptides were identified by LC-MS/MS sequencing (Sangon Biotech).

Construction of pGEX-4T-1-*nme2*, pGEX-4T-1-*rps3*, and pGEX-4T-1-*vimentin*

Total RNA was isolated from IPEC-J2 cells using TRIzol™ Reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, full-length cDNAs were generated by RT-PCR. 2 µg of total RNA prepared from cells was mixed with oligo(dT) primer, incubated at 72 °C for 10 min, and then reverse-transcribed at 42 °C for 90 min in a 25 µl reaction containing 5 µl of RT buffer, 5 µl of dNTPs (10 mM), RNA template (2 µg), 0.5 µl of reverse transcriptase, and RNase-free water. PCR was subsequently performed using 50 ng of cDNA, 10 µM forward and reverse primers, 25 µl PrimeSTAR® HS (Premix) (Takara Bio) and adjusted with nuclease-free water to a final volume of 50 µl. PCR cycling conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 10 min.

For construction of *nme2*, *rps3*, and *vimentin* into the pGEX-4T-1 expression vector (GE Healthcare), CDSs were amplified by PCR using gene-specific primers (Table 1). The amplified prod-

ucts and pGEX-4T-1 vector were digested with the respective restriction enzymes, gel-purified, and ligated overnight at 16 °C. The resulting plasmids, pGEX-4T-1-*nme2*, pGEX-4T-1-*rps3* and pGEX-4T-1-*vimentin*, were transformed into *E. coli* DH5 α . The correctness of these constructs was confirmed by PCR and DNA sequencing (Sangon Biotech).

Expression and purification of GST fusion protein

The three recombinant plasmids (pGEX-4T-1-*nme2*, pGEX-4T-1-*rps3*, and pGEX-4T-1-*vimentin*) were transformed into *E. coli* BL21 (DE3). To obtain soluble GST-tagged fusion proteins, cells were harvested by centrifugation and resuspended in lysis buffer (1 PBS, 0.1 mM PMSF, 1% Triton X-100). Following sonication, the cell lysate was centrifuged at 12,000 g for 30 min at 4 °C, and the supernatant was applied to a GSH-Sepharose 4B column (GE Healthcare). After washing the beads with 1 x PBS, GST fusion proteins were eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced GSH). The purified proteins were buffer-exchanged into GST pull-down binding buffer using Amicon Ultra 0.5-ml 10K filters and stored at -80 °C for subsequent use.

Expression and purification of TrxA-His-ENO2 fusion protein

The plasmid pET32a-*eno2* was transformed into *E. coli* BL21 (DE3). The transformed bacteria were cultured overnight, diluted 1 : 50 into fresh LB broth containing 100 μ g/ml ampicillin, and grown at 37 °C until an optical density of 0.6 was reached. Protein expression was induced with 0.4 mM IPTG for 7 h at 28 °C. Cells were harvested by centrifugation, resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.1 mM PMSF, 10% glycerol, 5 mM imidazole), and sonicated. The cell lysate was centrifuged at 12,000 g for 30 min at 4 °C, and the supernatant was loaded onto a Ni-NTA affinity column (Beyotime Biotechnology). The column was washed with 120 ml of washing buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.2 mM PMSF, 10% glycerol, 20 mM imidazole), and the His-tagged fusion protein was eluted with 10 ml of elution buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.1 mM PMSF, 10% glycerol, 200 mM imidazole). The purified fusion proteins were buffer-exchanged into GST pull-down binding buffer using Amicon Ultra 0.5-ml 10K filters and stored at -80 °C for subsequent use.

GST pull-down assay

The procedure for GST pull-down assay was performed as previously described (Chiu et al. 2018, 2019). Briefly, 2 μ g of purified GST or GST fusion proteins were coupled to GSH-Sepharose 4B beads (GE Healthcare) and incubated overnight at 4 °C on a horizontal shaker. The beads were then washed three times with 1 x PBS. GST-bound or GST fusion protein-bound beads were incubated overnight at 4 °C with 1 μ g of purified TrxA-His-ENO2 in pull-down binding buffer (20 mM Tris-HCl, 25 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, and 10 μ g/ml cocktail protease inhibitor), individually. After incubation, the beads were washed five times with binding buffer, and bound proteins were eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced GSH). Eluted samples were analysed by Western blotting using specific antibodies. A total of 10% of the input TrxA-His-ENO2 fusion protein was used as an internal control.

Western blot analysis

Total protein concentrations from cell lysates or purified proteins were quantified using a BCA assay. Eluted proteins from co-immunoprecipitation or GST pull-down assays were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE Healthcare). Target proteins were detected with specific primary antibodies, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive signals were visualised using BeyoECL Star (Beyotime Biotechnology) according to the manufacturer's protocol and captured with the ChemiDoc MP imaging system (Bio-Rad, USA).

Generation of anti-ENO2 monoclonal antibody

The *eno2* DNA fragment was amplified by PCR using primers ENO2-F2 and ENO2-R2 (Table 1). The amplified product was digested with NdeI and HindIII and ligated into the corresponding sites of the pET30a vector. The resulting construct, pET30a-*eno2*, was transformed into *E. coli* BL21(DE3) (Takara Bio). The recombinant protein was expressed, purified, and used to generate mouse anti-ENO2 monoclonal antibodies by Fitgene Biotechnology (Guangzhou, China).

Excystation of oocysts and releasing of sporozoites

Excystation was performed as previously described (Worliczek et al. 2013). Oocysts suspended in 5 ml of ddH₂O were treated with 0.24% NaOCl, washed with 1 x PBS, and then vortexed with 0.5 mm Precellys® glass beads (Peqlab, Erlangen, Germany) for 15 s. The oocysts were transferred to a new centrifuge tube containing 5 ml of sterile 0.4% (w/v) pepsin in HCl (Merck) and incubated for 2 h at 37 °C, followed by centrifugation at 1,000 g for 10 min. The supernatant was discarded, and the pellet was resuspended in a sterile mixture of 8% fresh porcine bile and 0.8% trypsin (w/v, Merck) in HBSS (Merck). The oocysts were incubated for an additional 2–3 h, with repeated microscopic monitoring, until most sporozoites were released. After another centrifugation at 1,000 g for 10 min, the pellet was resuspended in DMEM/Ham's F12 (Thermo Fisher Scientific Inc., Waltham, USA) containing 5% FCS. Free sporozoites were counted using trypan blue exclusion test.

Immunofluorescence assays (IFAs)

Indirect immunofluorescence assays were performed as previously described (Ray and Miller 1991) on both sporulated oocysts and sporozoites of *C. suis*. To examine CsENO2 distribution in host cells, sporulated oocysts and sporozoites were fixed on glass coverslips with 4% formaldehyde for 20 min at room temperature, blocked with SuperBlock T20 solution (Thermo Fisher Scientific Inc.) for 30 min at 37 °C, and then incubated with anti-ENO2 mAb (1 : 500) for 1 h at 37 °C. After washing, samples were incubated overnight at 4 °C in the dark with Alexa Fluor 488-conjugated donkey anti-mouse IgG (1 : 1000; Jackson ImmunoResearch, USA). Pre-immune serum was used as a negative control. Finally, samples were washed three times with 1 x PBS and visualised using the EVOS™ M7000 imaging system (Thermo Fisher Scientific).

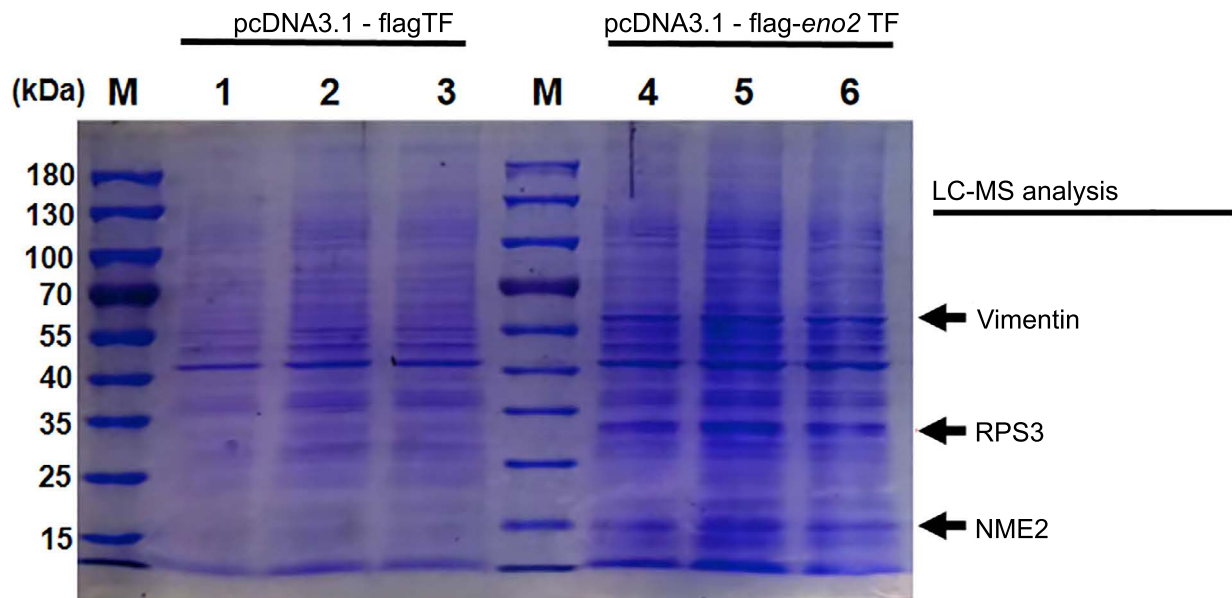


Fig. 1. SDS-PAGE analysis of cellular factors interacting with CsENO2 via Co-IP/LC-MS. IPEC-J2 cells were transfected with pcDNA3.1-flag or pcDNA3.1-flag-*eno2* plasmids for 24 hours, followed by a co-immunoprecipitation assay using a Flag monoclonal antibody. The co-immunoprecipitated proteins were separated by SDS-PAGE. Lanes 1–3 represented the pcDNA3.1-flag transfection, while lanes 4–6 represented the pcDNA3.1-flag-*eno2* transfection. TF: transfection.

RESULTS

Cellular proteins NME2, RPS3 and Vimentin coimmunoprecipitate with CsENO2

To identify potential cellular factors that specifically interact with CsENO2, IPEC-J2 cells were transfected with pcDNA3.1-flag-*eno2* for 24 h, followed by a co-immunoprecipitation assay using the Flag mAb. To obtain sufficient amounts of CsENO2-coimmunoprecipitated proteins for LC/MS analysis, cells from ten 10-cm culture dishes were harvested, lysed, and concentrated. Co-immunoprecipitated proteins were analysed by SDS-PAGE (Fig. 1). Three independent co-immunoprecipitation experiments revealed distinct and significant bands on SDS-PAGE. LC-MS analysis identified three host factors, NME2 (17 kDa), RPS3 (27 kDa) and Vimentin (55 kDa) that may coimmunoprecipitated with CsENO2 (Supplementary Table. S1). To further investigate the direct interaction between CsENO2 and these cellular proteins, GST pull-down assays were carried out.

Constructions of pET32a-*eno2*, pGEX4T-1-*nme2*, pGEX4T-1-*rps3*, and pGEX4T-1-*vimentin*

Full-length cDNAs were generated by RT-PCR from total RNA, and CDSs of NME2, RPS3, and vimentin were amplified from IPEC-J2 cells using the primers listed in Table 1. After confirmation by DNA sequencing, the amplified fragments and pET32a or pGEX-4T-1 vectors were digested with the restriction enzymes indicated in Table 1. The inserts and vectors were gel-purified and ligated overnight at 16 °C. The resulting plasmids were transformed into *Escherichia coli* DH5 α , and the constructs were verified by PCR and DNA sequencing.

TrxA-His-ENO2 is predominantly expressed in an insoluble form

Escherichia coli BL21 (DE3) carrying a plasmid encoding His-tagged ENO2 was induced with 1 mM IPTG for 3 h at 37 °C. Cells were pelleted, resuspended in 30 ml lysis buffer, and shaken at 4 °C for 30 min. Soluble and insoluble fractions were separated by centrifugation at 12,000 g for 30 min. The pellet was resuspended in 30 ml STE buffer (150 mM NaCl, 50 mM Tris, 1 mM EGTA). Protein concentrations in the whole extract, pellet, and supernatant were determined using a BCA assay. Aliquots of 20 μ g from each fraction were separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. A dense TrxA-His-ENO2 band was observed in the pellet, with little detected in the supernatant (data not shown), indicating that the protein was predominantly expressed in an insoluble form, likely as inclusion bodies.

Expression and purification of His-tagged and GST-tagged fused proteins

Escherichia coli BL21 (DE3) carrying plasmids encoding His- or GST-tagged fusion proteins was induced with 1 mM IPTG for 3 h at 37 °C. Following purification using affinity resins, GST-tagged NME2 and RPS3 were successfully obtained (Fig. 2B, C). In contrast, GST-Vimentin accumulated predominantly in the pellet, making it difficult to obtain sufficient amounts of soluble protein. Adjusting expression conditions to 16 °C for 16 h with 0.01 mM IPTG did not yield sufficient GST-Vimentin in the eluates (Fig. 2D), precluding subsequent GST pull-down assays. Similarly, TrxA-His-ENO2 expressed at 37 °C for 3 h was primarily found in the pellet. However, under the modified expression conditions at 16 °C for 16 hours with 0.01 mM

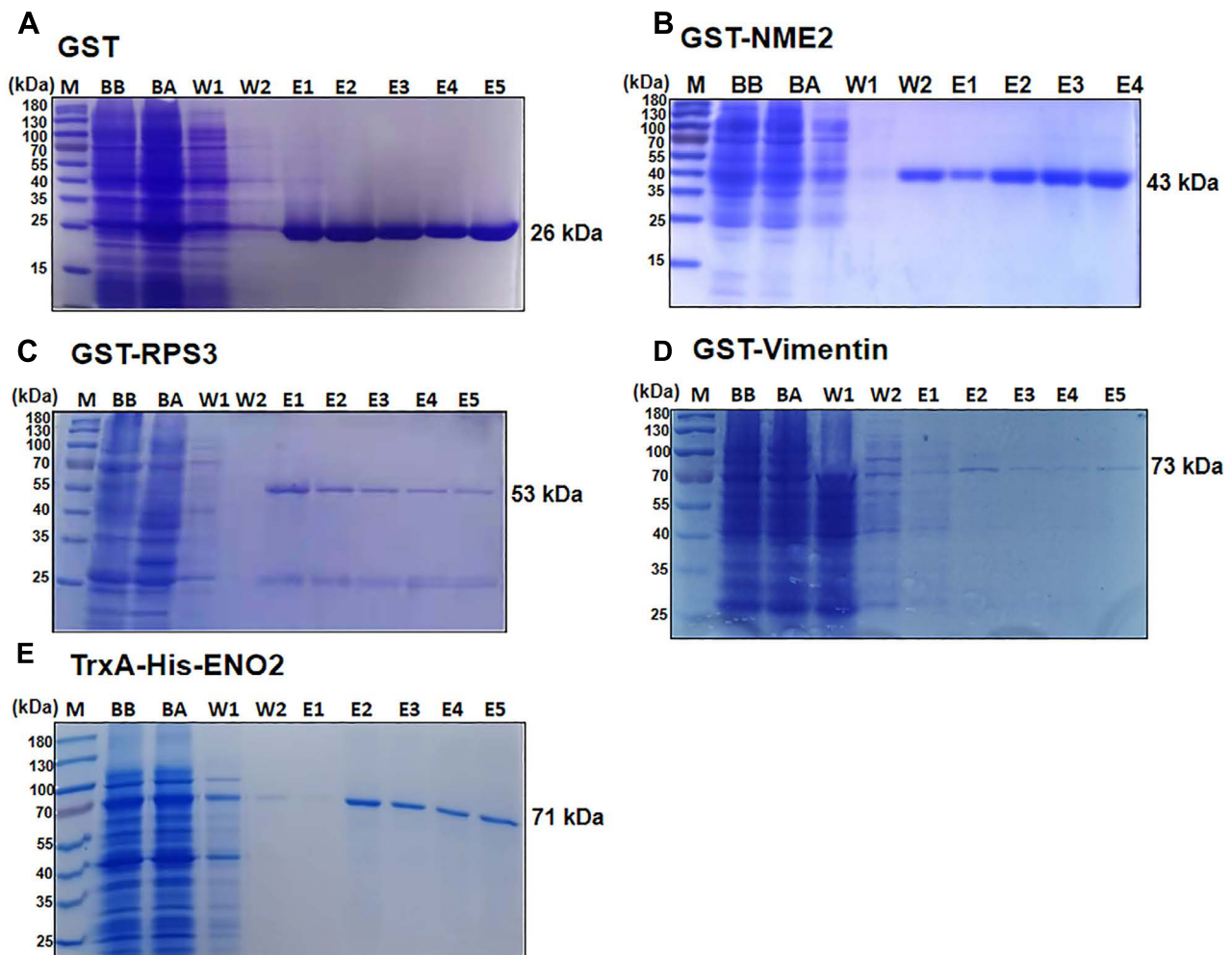


Fig. 2. SDS-PAGE analysis of purified His-tagged and GST-tagged fusion proteins. Lane assignments were as follows: BB – cell lysates binding before the column; BA – cell lysates binding after the column; W1–W2 – the fractions of the resins washed with washing buffer; E1–E5 – the fractions of the resins eluted with elution buffer. **A** – GST protein; **B** – GST-NME2 fused protein; **C** – GST-RPS3 fused protein; **D** – GST-Vimentin fused protein; **E** – TrxA-His-ENO2 fused protein.

IPTG, an improved soluble form of TrxA-His-ENO2 was successfully obtained in the supernatant and successfully purified (Fig. 2E).

GST pull-down assays

For GST pull-down assays, purified proteins were concentrated and buffer-exchanged into GST pull-down binding buffer using Amicon Ultra 0.5 ml 10K filters, as described previously (Chiu et al. 2018) (Fig. 3A–D). Western blot analysis of the eluted fractions revealed that NME2 and RPS3 directly interact with CsENO2 (Fig. 3E, F), consistent with the LC-MS results.

The distribution of CsENO2 in sporulated oocysts and sporozoites

No fluorescence was detected in sporulated oocysts or sporozoites when using mouse pre-immune serum as a negative control. Following incubation with the anti-ENO2 monoclonal antibody, CsENO2 was predominantly localised to the cytoplasm of sporulated oocysts. Additionally, staining of sporozoites revealed that CsENO2 was mainly

distributed in the nucleus, perinucleus, and extranuclear regions (Fig. 4).

DISCUSSION

Using Co-IP/LC-MS and GST pull-down assay, CsENO2, which is highly expressed in the sporulated oocysts of *Cystoisospora suis* (Yang et al. 2020), directly interacts with NME2 and RPS3 in IPEC-J2 cells. To our knowledge, this interaction between these two cellular proteins and ENO2 has not been previously reported. In addition, α -enolase has been shown to function as a *c-myc* promoter-binding protein (Ray and Miller 1991) and a strong plasminogen-binding protein (Miles et al. 1991, Nakajima et al. 1994, Pancholi and Fischetti 1998, Chen et al. 2002, Wygrecka et al. 2009, Deng et al. 2019).

Future studies combining co-immunoprecipitation and immunofluorescence staining (Chiu et al. 2018, 2019) could further investigate the binding motifs between NME2, RPS3, and CsENO2. Although vimentin was identified as a candidate interacting protein by LC-MS analysis, we were unable to obtain sufficient soluble protein

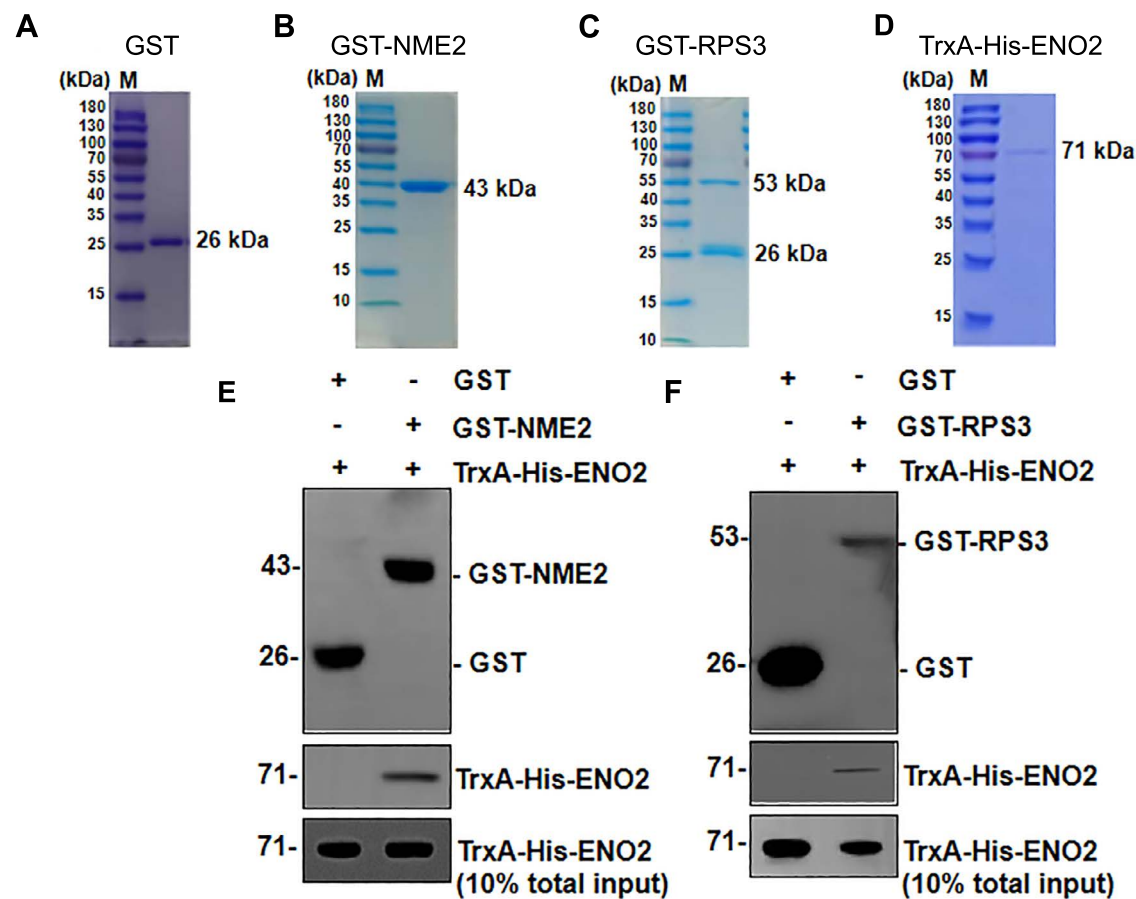


Fig. 3. Concentrated His- and GST-tagged fusion proteins and GST pull-down assay. Purified protein was concentrated and buffer-exchanged into GST pull-down binding buffer using a 10K filter (Amicon). Following concentration, 10 μ l of each sample was denatured and analysed by SDS-PAGE. Panels A–D show GST protein, GST-NME2, GST-RPS3, and TrxA-His-ENO2, respectively. An *in vitro* GST pull-down assay was conducted to assess whether ENO2 interacts with NME2 or RPS3, with GST served as the negative control. All proteins were analysed by Western blotting. 10% of the total input of TrxA-His-17 represented the loading control of p17 E, F.

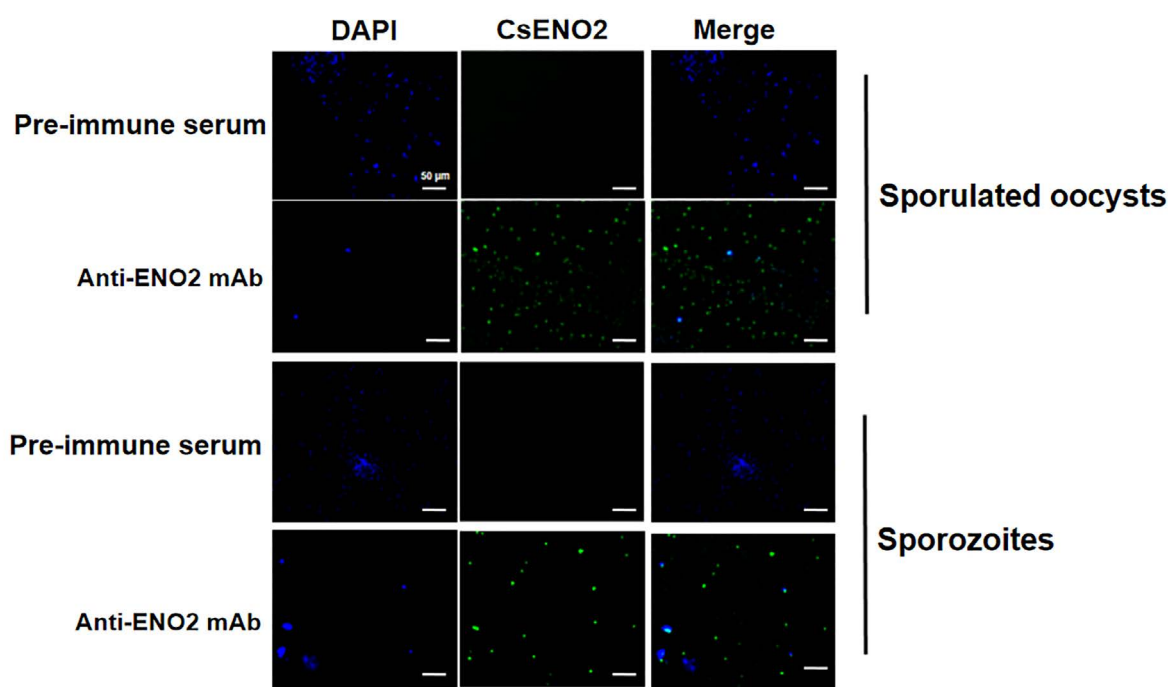


Fig. 4. The distribution of CsENO2 in sporulated oocysts and sporozoites was examined using indirect immunofluorescence assays. Pre-immune mouse serum served as the negative control for detecting CsENO2 in sporulated oocysts. Anti-CsENO2 monoclonal antibody was used to determine CsENO2 distribution in sporulated oocysts and sporozoites. Scale bar: 50 μ m.

for GST pull-down assays, representing a limitation of this study. Future use of the proximity ligation assay (PLA) may help visualise protein-protein interactions and address this issue (Sable et al. 2018, Alcalá et al. 2022). Additionally, the overexpression-based IPEC-J2 system may not fully reflect natural infection conditions; thus, *in vivo* studies using *C. suis*-infected host cells are needed to validate these protein interactions under natural conditions.

Enolase is a highly conserved multifunctional protein in eukaryotic and prokaryotic cells, with diverse roles in parasites, including its involvement in glycolysis (Zhou et al. 2020). It also serves as a plasminogen receptor on the cell surface of certain pathogens; for example, antibodies against enolase in *Leishmania mexicana* (Biagi, 1953) inhibit up to 60% of plasminogen binding (Vanegas et al. 2007). In *Plasmodium falciparum* Welch, 1897, neutralising surface enolase on merozoites blocks host cell invasion and transmission (Dutta et al. 2018). Accordingly, enolase can be localised in the nucleus, cytoplasm, or cell surface (Zhou et al. 2020).

In this study, fluorescence microscopy was used to examine the preliminary distribution of CsENO2 in sporulated oocysts and sporozoites. CsENO2 was found in the cytoplasm of sporulated oocysts, likely reflecting its glycolytic function, as reported in other parasites (Denton et al. 1996; Unterwieser et al. 2018; Zhou et al. 2020). Partial nuclear localisation was observed in sporozoites, suggesting a potential role in transcriptional regulation (Liu et al. 2016). In conclusion, this study identifies CsENO2-interacting proteins in IPEC-J2 cells and determine the localisation of CsENO2 within sporulated oocysts and sporozoites.

Authorship contribution statement. Hung-chuan Chiu: Writing–review & editing, Writing–original draft, Visualisation, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualisation. Yuhao Ao: Visualisation, Validation, Investigation. Yaoyue Tang: Writing–review & editing

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