Molecular Characterization and Serodiagnostic Potential of Two Serpin Proteins in Psoroptes Ovis

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Abstract

**Background:** *Psoroptes ovis* a common ectoparasite of wild and domestic animals, leads to cutaneous inflammation, extreme pruritus, scaly lesions, and causes an economically devastating loss of animal husbandry and animal welfare issues. Serine proteinase inhibitor (Serpin) is present in almost all organisms that are involved in host-pathogen interactions, inflammatory responses, and reproductive development, etc. However, the research on *P. ovis* serpins is still limited.

**Methods:** In this study, two serpins of *P. ovis* (PsoSP1 and PsoSP2) were cloned, and the molecular characterization was analyzed by bioinformatics. The transcriptional profiles and tissue localization of PsoSP1 and PsoSP2 in *P. ovis* were investigated by quantitative real-time PCR and immunohistochemistry, respectively. The potential function of recombinant PsoSP1 and PsoSP2 (rPsoSP1 and rPsoSP2) in the serodiagnosis of *P. ovis* infestation in rabbits were evaluated by indirect enzyme-linked immunosorbent assay (iELISA).

**Results:** Both of the 523 residue PsoSP1 and the 240 residue PsoSP2 proteins contained typical serpin domains and signatures. Both PsoSP1 and PsoSP2 expressed throughout the life cycle, more specifically, significantly higher expression in female mites than the larva, nymph, and male mites (*p* < 0.001). The native PsoSP1 and PsoSP2 proteins localized in ovary and mouthpart of adult female mites, respectively. Compared to rPsoSP2, the rPsoSP1 displayed better diagnostic efficiency with higher values of sensitivity, specificity and the area under the receiver operating characteristic curve (AUC) than rPsoSP1 by iELISA (rPsoSP1 - rPsoSP2: 96.0% - 90.0%; 90.91% - 78.18%; 0.988 - 0.964, respectively). Moreover, the rPsoSP1 showed seropositive in 80% rabbits as early as the 2nd week post-infestation (p.i.), prior to visible clinical signs and microscopy-positive of skin scrapings.

**Conclusions:** These results suggested that these two serpins may play essential roles in reproductive development, blood-feeding, and pathogenicity of *P. ovis*. Compared to PsoSP2, PsoSP1 appeared as a potential antigen for serodiagnosis of *P. ovis* infestation in rabbits, especially at the early stage of infestation.

**Introduction**

*Psoroptes ovis* is a common ectoparasite of wild and domestic animals worldwide and is divided into subspecies according to the name of its host, e.g., *P. ovis* var. *cuniculi* from rabbits, *P. ovis* var. *bovis* from cattle [1]. This mites cause various features of the psoroptic disease, mainly characterized as intense cutaneous inflammation, extreme pruritus and crusted skin lesions [2, 3]. Additionally, it causes severe economic losses and animal welfare issues, especially in sheep, rabbits, and cattle [3, 4, 5].

The obligate ectoparasite *P. ovis*, as a non-burrowing mite, spends its entire life on the surface of host skin. The life cycle lasts 10 ~ 19 days from egg hatch to egg production in adults [6]. *P. ovis* feeds on serous fluids, lymph, and red blood cells [7]. Consequently, mite produces essential proteins to resist the host hemostatic system for successful blood feeding and self-proliferation. Meanwhile, it excretes
allergens to promote the subsequent cutaneous inflammatory response [8, 9]. Serine protease inhibitor (serpin), expressed in almost all organisms, has shown a variety of fundamental physiological functions in arthropods including anticoagulation, regulation inflammation response and reproductive development etc. [10]. It also played an essential role in host-pathogen interaction [11]. Besides, serpin may serve as a promising diagnostic antigen or vaccine candidate [12, 13].

Recently, genome and transcriptome analyses revealed that serpins existed in P. ovis [14, 15], but beyond that, no research has been reported on P. ovis serpins. Therefore, we are highly interested in the function of two serpins of P. ovis from rabbits (also known as Psoroptes cuniculi) (PsoSP1 and PsoSP2) which were identified based on our transcriptomic data [14]. In this study, we cloned and expressed the two recombinant PsoSP1 and PsoSP2 in prokaryotic expression vectors, performed the bioinformatics analysis. Additionally, we also investigated the transcriptional profiles as well as tissue localization in mites, and their potential efficiencies in the diagnosis of P. ovis infestation in rabbits were accessed by indirect enzyme-linked immunosorbent assay (iELISA). This is a preliminary study in relevance to the roles of these two proteins in P. ovis, that laying the foundation for further understanding of their functions.

Materials And Methods

Mite collection and RNA extraction

P. ovis var. cuniculi were harvested from an infested New Zealand White rabbit maintained at the Department of Parasitology, Sichuan Agricultural University (Sichuan, China). About 300 mites, a pool of larvae, nymphs and adults, were collected and processed for the total RNA extraction using a MiniBest universal RNA extraction kit (TaKaRa, Dalian, China).

Cloning of two PsoSP genes

Total RNA was converted into cDNA following the protocols of the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The PsoSP1 and PsoSP2 genes were amplified from cDNA by using the following primers: 5’-CGGGATCCGCTCATGTTGGGCTCAACCATC-3’ (forward) and 5’ CCAAGCTTTTTAAAAACATGAATTTCACC-3’ (reverse) for PsoSP1 with underlined restriction enzymes of BamHI and HindIII, and 5’-CGGGATCCGCTCATGTTGGGCTCAACCATC-3’ (forward) and 5’-CCCTCGAGTCAAAATCCATGCATTTCACC-3’ (reverse) for PsoSP2 with underlined restriction enzymes of BamHI and XhoI. The DNA fragments were purified and ligated into the pMD19-T (TaKaRa, Dalian, China). Then, the plasmids were transformed into Escherichia coli strain DH5α (TIANGEN Biotech Co. Ltd., Beijing, China), and sequences were confirmed by Invitrogen Biotechnology Company (Shanghai, China).

Sequence analysis

DNAMAN version 7.0 was applied to compare the similarity between paralogous genes, and SignalP 5.0 (http://www.cbs.dtu.dk/Services/SignalP/) was used to predict signal peptides. Transmembrane regions were analyzed using the Transmembrane Prediction Server (http://www.cbs.dtu.dk/services/TMHMM-
2.0). B-cell epitopes were predicted by BaCelLo (http://gpcr.biocomp.unibo.it/bacello/pred.htm). The serine protease inhibition domains were analyzed by Inter-ProScan EMBL-EBI Software (http://pfam.xfam.org/). Amino acid sequences were aligned using MEGA5.0. Secondary structure predictions were performed by JPred 4.0 (http://www.compbio.dundee.ac.uk/jpred/). The neighbor-joining (NJ) tree, including values of 1000 replications resampled tests, was constructed by MEGA 5.0 software [16].

**Expression and purification of PsoSP**

The correct plasmids of two PsoSP genes were digested and ligated into the pET32a (+) expression vector (Invitrogen, Beijing, China), respectively. Then, the resulting constructs were transfected into E. coli BL21 (DE3) and induced by 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) at 20 °C for 10 h. The recombinant PsoSP1 (rPsoSP1) and PsoSP2 (rPsoSP2) were harvested from the supernatant and inclusion body, respectively, and further purified by a Bio-scale™ Mini Nuvia™ IMAC Ni-Charged column (Bio-Rad, California, USA) using a step-wise elution with 50, 100, 200, 300 and 400 mM imidazole. The eluted fractions were concentrated by Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA).

**Rabbit sera**

Fifty *P. ovis* var. *cuniculi*-positive rabbit sera were collected from a farm located in Chengdu, Sichuan, China. All rabbits were confirmed *P. ovis* var. *cuniculi*-positive by observation of ear scab and skin scrapings by microscopy (Ochs et al., 2001). Twenty-five negative sera from *P. ovis* var. *cuniculi*-free rabbits were obtained from a farm without a history of psoroptic mange. For cross-reaction testing, another 30 sera included *Sarcoptes scabiei*-positive sera, *Eimeria* spp.-positive sera, and *Cysticercus pisiformis*-positive sera (ten/group) were provided by the Department of Parasitology, Sichuan Agricultural University.

**Preparation of polyclonal antibodies and Western blotting**

Polyclonal antibodies of anti-rPsoSP1 and anti-rPsoSP2 from rabbits were obtained by experimentally immunized with purified rPsoSP1 and rPsoSP2, respectively. The rabbits were immunized with about 1 mg purified recombinant protein mixed with an equal volume of Freund’s complete adjuvant (Sigma-Aldrich) by subcutaneous injection at day 0. The next two immunizations were performed with mixture 1 mg protein and the same amounts of Freund’s incomplete adjuvant at days 7 and 14, respectively. At 7 days after the final immunization, sera were collected from rabbits via the marginal ear vein, and then purified by HiTrap Protein A affinity chromatography (Bio-scale™ Mini UNOsphere SUPrA™ Cartridge) to obtain the IgG of anti-rPsoSP1 and anti-rPsoSP2.

Two purified rPsoSP proteins were separated by 12% SDS-PAGE and transferred to the nitrocellulose membranes using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The membranes were blocked using 5% skim milk powder for 2 h. After three times of 5-min washes with TBST (0.02 M Tris-HCl, pH 7.6, 0.15 M NaCl, 0.05% Tween-20), membranes were incubated with rabbit anti-*P. ovis* var. *cuniculi* antibody
or anti-rPsoSP1 IgG or anti-rPsoSP2 IgG (1:150 v/v) overnight at 4 °C. Non-infested rabbit serum was used as a negative control. After washing three times with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:1000 dilution; Boster Bio-project Co. Dalian, China) for 1 h at room temperature. Following three washes with TBST, the signal was detected using an Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen, Beijing, China).

**Immunolocalisation of PsoSP1 and PsoSP2 in adult female P. ovis var. cuniculi**

Adult female mites were fixed in 0.8% (w/v) agarose, and then soaked in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h to investigate the tissue localization of PsoSP1 and PsoSP2. The mites were processed according to the paraffin-embedded tissue technique and sliced into 5 µm histological section using a rotary microtome (Leica, Frankfurt, Germany). After dewaxing and rehydration, parts were treated with 0.01 M citrate buffer and incubated at 37 °C with 5% (w/v) skimmed milk for 4 h, then incubated with purified rabbit anti-rPsoSP1 IgG or anti-rPsoSP2 IgG or pre-immune IgG (1:200 v/v) overnight at 4 °C. After three times of 5-min washing with PBS, sections were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200 v/v) at 37 °C for 1 h in the dark and finally visualized by a fluorescent microscope (BX53, Olympus, Japan).

**Transcriptional profiles of PsoSP at different life stages of P. ovis var. cuniculi**

To analyze stage-specific gene expression profiles of PsoSP1 and PsoSP2 in *P. ovis var. cuniculi*, the total RNA was extracted from larvae, nymph, and adult (male/female) mites using a MiniBest universal RNA extraction kit (TaKaRa), respectively. Relative gene expression was evaluated by two-step qRT-PCR with the following primers: PsoSP1, 5'-TGGCAGCAGTGGATCAGAATCATC-3' (forward) and 5'-AATGCAACAGCAACACTGTATGGC-3' (reverse); PsoSP2, 5’-TCCTACATACACGTCCATAACGCGGCTG-3’ (reverse). The β-actin gene was used as a housekeeping control to correct the relative fluorescence signal of the target genes using primers 5'-TGAATTGCTGATGGTCAAG-3' (forward) and 5'-TGGCGAACAAGTCTTTACGG-3 (reverse). Gene transcription was assessed according to the manufacturers’ recommendations of the real-time PCR System (LightCycler® 96 System, Roche, Switzerland) and the SYBR Premix Ex Taq II Kit (TaKaRa, Dalin, China). The qPCR experiments were carried out in 25 µL reactions containing 12.5 µL SYBR premix Ex Taq II, 1 µL of each primer, 2µL cDNA and 8.5 µL ddH₂O. Each sample was performed in triplicate. An equal volume of ddH₂O replaced the template cDNA as blank control. Thermal cycling was performed as follows: 95 °C for 30 s, 95 °C for 5 s, 58 °C for 30 s, then followed by 40 cycles at 95 °C for 0 s, 59 °C for 15 s, and 95 °C for 0 s. Melting curves were plotted, and relative expression levels of the target genes were calculated by the 2−ΔΔCt method.

**Establishment of an Indirect ELISA (iELISA)**
The establishment of iELISA was performed as described by Crowther and Walker [17]. The concentrations of antigen and primary serum samples were determined by the checkerboard titration tests. Briefly, the purified proteins were diluted two-fold in 0.1 M carbonate buffer (pH 9.6) to the different concentrations and coated in 96-well plates with 100 µL/well overnight at 4 °C. The dishes were washed three times with PBS containing Tween-20 (PBST, pH 7.4) (5 min per wash), then incubated with 5% (w/v) skim milk powder at 37 °C for 90 min. After washing three times, 100 µL of two-fold gradient dilution of *P. ovis* var. *cuniculi*-positive and negative serum samples (diluted in PBS ranging from 1:20 to 1:320) were added and incubated at 37 °C for 1 h. The plates were washed 3 times with PBST and incubated 1 h at 37 °C with 100 µL HRP-labeled goat anti-rabbit IgG (1: 3000 dilution with 0.01 M PBS) (Boster Bio-project Co., Wuhan, China). After 4 times washing with PBST, 100 µL of TMB chromogenic solution (TIANGEN, Beijing, China) was added at 37 °C for 20 min, then the reaction was stopped with 100 µL / well of 2 M H$_2$SO$_4$. Optical densities (OD) were read at 450 nm by a microplate reader (Thermo Scientific, Pittsburgh, PA, USA). The optimal working conditions were determined with the highest P/N (positive/negative serum) value. The cut-off value of iELISA was determined as the mean OD450 value plus three standard deviations (SD) using 25 negative serum samples from naïve rabbits [1].

To further evaluate the feasibility of the iELISA, 50 *P. ovis* var. *cuniculi*-positive serum samples were assessed by the iELISA, and the sensitivity was calculated as (ELISA positive × 100) / true *P. ovis* var. *cuniculi*-positive [1]. Thirty serum samples from rabbit infected with *S. scabiei*, *Eimeria* spp and *C. pisiformis* (10 samples for each species) were used to evaluate the cross-activity of the iELISA. Twenty-five negative serum samples from naïve rabbits and 30 serum samples in the cross-activity assay were used to determine the specificity of the iELISA, which was calculated as (ELISA negative × 100) / real *P. ovis* var. *cuniculi*-negative [1]. After that, the area under the receiver operating characteristic curve (AUC), a graph of the sensitivity (true positive rate) versus 1-specificity (false positive rate), was calculated by MedCalc 19.0.7 [18].

The repeatability (intra-assay variability) and reproducibility (inter-assay variability) of the iELISA were evaluated using three *P. ovis* var. *cuniculi*-positive serum samples, substantially as previously described [19].

**The experimental infestation of rabbits with *P. ovis* var. *cuniculi* and serological testing using the established iELISA**

Rabbits infected with *P. ovis* var. *cuniculi* were performed strictly as previously described [3]. Briefly, ten 3-month-old naïve New Zealand rabbits (5 females and 5 males) were infested on the aural region with approximately 200 mites of mixed stages of *P. ovis* var. *cuniculi*. Three non-infested rabbits were applied as controls. Serum samples from 13 rabbits were collected from the marginal ear vein at weeks 0, 1, 2, 3, and 4. Afterward, in a total of 65 serum samples (50 from the *P. ovis* var. *cuniculi* infestation rabbits and
15 from the non-infestation rabbits) were examined by the establishment optimal iELISA method. Each serum sample in triplicate was analyzed in one ELISA plate, and positive and negative controls were contained in the dishes.

**Statistical analysis**

All data are represented as mean ± standard deviation (SD), and statistical differences between groups were evaluated using Mann-Whitney U tests in SPSS software v.17.0. *P*-values < 0.05 were considered as statistically significant.

**Results**

**Bioinformatics analyses of two PsoSP**

The 1572-bp open reading frame (ORF) in the cDNA sequence of PsoSP1 (GenBank: MT707535) encodes 523 amino acids (aa), while the 723-bp ORF in the cDNA sequence of PsoSP2 (GenBank: MT707536) encodes 240 aa. The PsoSP1 protein contains a signal peptide at an amino acid position from 1 to 17 but no transmembrane region, whereas PsoSP2 protein appears to contain no signal peptide and a transmembrane region.

PsoSP1 and PsoSP2 shared 31.33% ~ 50.85% and 28.99% ~ 69.92% amino acid sequence identity with orthologs in *Dermatophagoides farinae*, *D. pteronyssinus*, *Euroglyphus maynei*, and *Sarcoptes scabiei* (Fig. 1). Secondary structure prediction showed that PsoSP1 protein was comprised of 12 helix and 3-sheets, and PsoSP2 protein contained 5 helices and 3-sheets (Fig. 1a). A serpin domain was identified in the amino acid sequence from Arg78 to His493 for PsoSP1 and Asn4 to Met237 for PsoSP2 [20]. Moreover, both two proteins appeared to possess the specific serpin signature at the deduced amino acid sequences from 496 to 506 (LRFDHPFLYFV) for PsoSP1 and from 213 to 223 (LSFDHPFLYFL) for PsoSP2, respectively (Fig. 1a and 1b). The NJ tree revealed that PsoSP1 had a close relationship with *D. farinae*-Der f 27 allergen, *D. pteronyssinus*-Der f 27-like allergen, and *E. maynei*-Serpin, whereas PsoSP2 was grouped with *E. maynei*-Serpin-like and *D. pteronyssinus*-Serpin B10-like (Fig. 2).

**Acquisition of recombinant PsoSP1 and PsoSP2**

The rPsoSP1 were mainly present in the supernatant with a single band of ~ 75 kDa, whereas rPsoSP2 principally present in insoluble inclusion bodies with the single bands of ~ 46 kDa (including ~ 18 kDa His-tag fusion peptide from pET-32a) (Fig. 3).

**Western blotting**

The rPsoSP1 and rPsoSP2 were reacted with *P. ovis* var. *cuniculi*-positive sera and the correspondent antisera IgG from rabbits, but not negative sera, revealing the favorable reactivity and antigenicity (Fig. 3).
Tissue localization of PsoSP1 and PsoSP2 in adult female
*P. ovis* var. *cuniculi*

Immunofluorescence histochemistry indicated that native PsoSP1 and PsoSP2 were located in ovary and
mouthpart of female mites, respectively (Fig. 4b and 4c). No fluorescence signal was observed in adult
female mites using pre-immunized rabbit IgG antibodies (Fig. 4a).

Transcriptional profiles of PsoSP1 and PsoSP2 in *P. ovis*
var. *cuniculi*

Transcriptional profiles of PsoSP1 and PsoSP2 in different development stages of *P. ovis* var. *cuniculi*
were investigated by qRT-PCR (Fig. 5). Both PsoSP1 and PsoSP2 were expressed throughout the life cycle
of mites, more specifically, significantly higher expression in female mites than larva, nymph, and male
mites (*p* < 0.001) (Fig. 5).

Serodiagnosis potential of rPsoSP

By checkerboard titration, the optimal working conditions of iELISA were 46.0 µg/mL of rPsoSP1,
64.5 µg/mL of rPsoSP2 for coated antigens and a 1:100 dilution for rabbit sera. The cut-off values were
0.633 of rPsoSP1 and 0.490 of rPsoSP2, respectively.

The sensitivities were determined as the results of positive sera with 96.0% of rPsoSP1 (48/50) (Fig. 6a)
and 90.0% of rPsoSP2 (45/50) (Fig. 6b). The specificities showed 90.91% of rPsoSP1 (50/55) and
78.18% of rPsoSP2 (43/55). Consequently, the AUC was 0.988 of rPsoSP1-iELISA (95% confidence
interval (CI), 0.944 ~ 0.999) and 0.964 of rPsoSP2-iELISA (95% CI, 0.908 ~ 0.991), indicating that the
rPsoSP1-iELISA showed a better accuracy to detect specific antibodies against *P. ovis* var. *cuniculi*
than rPsoSP2-iELISA (Fig. 7). The AUC of rPsoSP1-iELISA was closed to 1, which indicated the effective
diagnostic accuracy. The intra- and inter-assay variabilities of rPsoSP1-iELISA were < 5%, indicating the
established rPsoSP1-iELISA was stable and reproducible.

Serodiagnostic test of rabbits experimentally infested with
*P. ovis* var. *cuniculi*

After 4 weeks of post-infestation (p.i.), all infested rabbits were observed with the visible ear scabs.
Meanwhile, *P. ovis* var. *cuniculi* showed positive in skin scrapings. By rPsoSP1-iELISA, the mean value of
the anti-rPsoSP1 level from the infestation group revealed an increase from 1 to 4 weeks p.i (Fig. 8). The
positive anti-rPsoSP1 above cut-off value was firstly detected with 2/10 serum samples at 1-week p.i. in
the infestation group. Afterward, the rate of positive serum gradually increased to 80% (8/10) at 2 and 3
weeks p.i., then up to 100% (10/10) at 4 weeks p.i. (Fig. 8). In the non-infestation group, the anti-rPsoSP1 antibody appeared below the cut-off value throughout the experiments.

Discussion

In the present study, two *P. ovis* serpins were characterized, and the potential of the recombinant proteins was evaluated for serodiagnosis of *P. ovis* infestation in rabbits. The predicted amino acid sequence showed the low overall identity of serpins compared to other mites (31.33% ~ 50.85% of PsoSP1 and 28.99% ~ 69.92% of PsoSP2), however, these two target proteins were identified as typical serpins due to the presence of the features such as serpin domain and serpin signature in C-terminal end [21]. PsoSP1 shared the highest amino acid sequence identity (50.85%) with the newly characterized *D. farinae* Der f 27 allergens, which has been proven to orchestrate the pulmonary inflammatory response and mediate Th2 type response in mouse [22]. Besides, NJ analysis revealed that PsoSP1 yielded a close relationship with Der f 27. In combination with the homology and the genetic relationship between Pso SP1 and Der f 27, PsoSP1 may be considered as an allergen of *P. ovis*, which was possibly associated with the instigation of the host cutaneous pro-inflammatory response [23]. Additionally, this cutaneous inflammation resulted in serum extravasation to provide sufficient food for mites population growth and cause aggravation of scabby lesions [7, 24]. The expression of PsoSP1 and PsoSP2 in all stages of mites indicated that PsoSP1 and PsoSP2 possibly play an essential role in the development of *P. ovis*. However, significant differences were seen for the transcription of PsoSP1 in female mites being the highest level of expression with a 347-fold change. In addition, the native protein was located in the ovary of female mites, indicating that PsoSP1 possibly was essential in vitellogenesis [15, 25]. This role of serpin being involved in vitellogenesis has been proven in a recent research, which indicated RNAi of serpin gene resulted in a reduction of yolk granule accumulation in *Rhipicephalus haemaphysaloides* [26]. In addition, previous studies have shown that serpins of ticks took part in anti-coagulation [27, 28], and were considered as the vaccine candidate against tick infestation [27] [13]. In this study, the localization of native PsoSP2 in mouthpart of female mites and the expression of PsoSP2 throughout the life stages of mites suggested that PsoSP2 may appear to be vital in mites for anti-coagulation to successful blood-feeding [7, 11]. The similar obligate hematophagous ectoparasites of ticks and mites indicate that PsoSP2 could serve as a potential vaccine candidate or therapeutic target as the alternative for current chemical acaricides.

Psoroptic mange spreads rapidly under crowded conditions and causes major morbidity in the rabbit breeding industry in China [29], resulting in an economically devastating loss. Thus, timely diagnosis and treatment of *P. ovis* infestation in rabbits are paramountly important to reduce the risk of disease transmission and improve profitability. In China, the current microscopic diagnosis for this disease is extremely time-consuming and inefficient in the low mite carriers and sub-clinical infestations in rabbits. Thus, it is imperative to seek for the effectively immunoreactive antigens for rapid and accurate diagnosis of the *P. ovis* infestation in rabbits. Furthermore, animals infested *P. ovis* could evoke sero-specific antibody [2, 30], and this sero-specific antibody was induced at the early phase of parasite infestation when animals appeared asymptomatic [2, 3, 31]. Thus, enzyme-linked immunosorbent assay
(ELISA) can be considered as an accurate method compared to the microscopy of skin scrapings at the low mite carriers and sub-clinical infestations because of its speedy, high sensitivity, and handling convenience. In a previous study, serpin of *Schistosoma mansoni* was considered as a promising diagnostic antigen in human schistosomiasis [12]. Therefore, in this study, we evaluated the serodiagnostic potential of rPsoSP1 and rPsoSP2 by the establishment of the iELISA. Compared to rPsoSP2-iELISA, the rPsoSP1-iELISA displayed better diagnostic efficiency with higher values of sensitivity, specificity and AUC (rPsoSP1 - rPsoSP2: 96.0% - 90.0%; 90.91% - 78.18%; 0.988 - 0.964, respectively). Although rPsoSP1 showed cross-reaction with sera from 3/10 *S. scabiei*-infestation, the cross-reaction between these two ectoparasites have been proved commonly in other studies [3, 32, 33]. Fortunately, these two mite species were effectively treated with the same acaricide [34, 35]. Besides, 1/10 rabbits infested with *C. pisiformis* and *Eimeria* spp. showed sero-reaction with rPsoSP1, however, their OD values were close to the cut-off value and appeared markedly lower than those rabbits infested with *P. ovis* var. *cuniculi* (p < 0.001). Moreover, rPsoSP1-iELISA can detect seropositive in 80% (8/10) rabbits as early as the 2nd-week p.i., prior to visible clinical signs and microscopy-positive of skin scrapings. Regarding the high sensitivity and specificity, PsoSP1 was more suitable as a candidate antigen for serodiagnosis of *P. ovis* infestation in rabbits, especially at the early stage of infestation.

**Conclusions**

In conclusion, PsoSP1 and PsoSP2 displayed the typical characterization of serpin superfamily with the regular serpin domain and signature. The expression of PsoSP1 and PsoSP2 were found in all life stages of mites, with significantly high expressions in adult female mites. Compared to rPsoSP2, rPsoSP1 seemed to display a better diagnostic efficiency than PsoSP2 by iELISA, suggesting that PsoSP1 could be developed as a potential antigen for serological diagnosis of *P. ovis* infestation in rabbits, especially at the early stage of infestation.

**Declarations**

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Authors contributions

XBG conceived and designed the experiments. YHC and CYZ performed the experiments including RT-PCR, qRT-PCR, recombinant protein expression, indirect ELISA. NXS and CW performed the tissue localization. XBG, YHC, YX, RH and XZ achieved the data analysis. GYY, XRP, DYY and ZH contributed reagents/materials/analysis tools. ZH and ZJZ provide experimental technical assistance. XBG and YHC wrote the initial manuscript. All authors critically revised and approved the final version of the manuscript.

Ethics approval and consent to participate

In this study, the animal procedure was reviewed and approved by the Animal Care and Use Committee of Sichuan Agricultural University (SYXX 2019-187). All the rabbits were strictly managed under the Guide for the Care and Use of Laboratory Animals (National Research Council, Bethesda, MD, USA) and the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures
Figure 1
Multiple sequence alignment of PsoSP1(a) and PsoSP2 (b). a: PsoSP1: Multiple sequence alignment of the deduced amino acid sequence of PsoSP1 with homologous sequences of related proteins of other parasites: Dermatophagoides farinae (GenBank: AIO08851.1), D. pteronyssinus (GenBank: ATI08940.1), Euroglyphus maynei (GenBank: OTF72764.1) and Sarcoptes scabiei (GenBank: AEB40052.1). b: PsoSP2: Multiple sequence alignment of the deduced amino acid sequence of PsoSP2 with homologous sequences of related proteins of other parasites: Euroglyphus maynei (GenBank: OTF74296.1), Dermatophagoides pteronyssinus (GenBank: XP_027200949.1), Sarcoptes scabiei (GenBank: KPM10873.1) and Dermatophagoides farinae (GenBank: AAP35082.1). Helices are marked as red tubes, and sheets as dark green arrows on the sequence. Elements of secondary structure are labeled as follows: (hA, hB, etc.) A-helix, B-helix, etc.; (s1A, s2A, etc.) strand 1 of the A β-sheet, strand 2 of the A β-sheet, etc. Consistent residues are highlighted with a yellow background, and consistent partial residues are highlighted with a brown background. B-cell epitopes are marked with a black box.

Figure 2

The neighbor-joining (NJ) tree was constructed based on the deduced amino acid sequence of serpin. The number beyond every branch is the bootstrap value of 1000 replications (%).
Figure 3

Immunoblotting with the serum binding recombinant PsoSP1 and PsoSP2 proteins. Lane M: protein molecular weight marker; Lane 1: recombinant proteins of E. coli expressing pET32a (+)-PsoSP1 induced by IPTG; Lane 2: recombinant proteins of E. coli expressing pET32a (+)-PsoSP2 produced by IPTG; Lane 3: the proteins of E. coli expressing pET32a (+); Lane 4: the purified rPsoSP1; Lane 5: the purified rPsoSP2; Lane 6: the purified rPsoSP1 immunoblotted with the positive serum of the rabbits with Psoroptic mange; Lane 7: the purified rPsoSP2 immunoblotted with the positive serum of the rabbits with Psoroptic mange; Lane 8: the purified rPsoSP1 immunoblotted with the anti-rPsoSP1 IgG; Lane 9: the purified rPsoSP2 immunoblotted with the anti-rPsoSP2 IgG; Lane 10: the purified rPsoSP1 immunoblotted with the negative serum; Lane 11: the purified rPsoSP2 immunoblotted with the negative serum.
Figure 4

Immunolocalization of PsoSP1 and PsoSP2 in the adult female of Psoroptes ovis var. cuniculi. a: Incubated with the negative IgG of the rabbit before immunization; b: Incubated with the specific IgG of anti-rPsoSP1; c: Incubated with the specific IgG of anti-rPsoSP2. All images were taken under a fluorescent microscope at the magnification of 100×.

Figure 5

Relative transcriptional profiles of PsoSP1 (a) and PsoSP2 (b). The internal reference gene was β-actin in the study. Data are represented as the mean with standard deviation (SD) in triplicate (*** indicates the statistical significance of p < 0.001).
Figure 6

Specificity, sensitivity and cross-reactivity of rPsoSP1 (a) and rPsoSP2 (b) by indirect ELISA. The thin horizontal line represents the cut-off value (rPsoSP1-iELISA: 0.633; rPsoSP2-iELISA: 0.490). Statistically significant differences were compared between P. ovis-positive serum and the other serum samples, including Eimeria spp, C. pisiformis, S. scabiei-positive, and P. ovis-negative serum samples (** indicates the statistical significance of p < 0.001).
Figure 7

The Receiver operating characteristic (ROC) curves of the rPsoSP1-iELISA and rPsoSP2-iELISA for the detection of antibodies against *P. ovis* var. cuniculi. The ordinate represents the sensitivity of the iELISA. The abscissa represents the 1-specificity of the iELISA. The purple line shows the mean area under the curve (AUC) plot of rPsoSP1-iELISA, and the green line shows the mean area under the curve (AUC) plot of rPsoSP2-iELISA.
Figure 8

Serum antibody profiles detected by rPsoSP1-iELISA in rabbits experimentally infected with P. ovis var. cuniculi. The ordinate represents the OD450 value of serum. The abscissa represents the serum of different infection period. The thin horizontal line represents the cut-off value (rPsoSP1-iELISA: 0.633).