In vitro evaluation of a cysteine protease from poultry red mites, *Demanyssus gallinae*, as a vaccine antigen for chickens

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ABSTRACT Poultry red mites (**PRMs**, *Dermanyssus gallinae*) are hematophagous ectoparasites that negatively affect egg production, which causes serious economic losses to the poultry industry worldwide. Currently, the emergence of acaricide-resistant PRMs has impeded PRM control in poultry farms. Several alternatives for acaricide use have been described for managing PRM-caused problems. Vaccination is among the methods for controlling PRMs in poultry houses. Currently, several candidates for vaccine antigens have been identified. This study identified a cysteine protease, Deg-CPR-2, which differs from 2 other previously reported cysteine proteases in PRMs, from previously obtained data from RNA-sequencing (**RNA-seq**) analysis. We investigated the characteristics of Deg-CPR-2 and assessed its efficacy as a vaccine antigen in vitro. Phylogenetic analysis revealed that Deg-CPR-2 belonged to a different cluster from those of other cysteine proteases in PRMs. This cluster also included cathepsin L-like proteases, enzymes thought to be involved in hemoglobin digestion in ticks. Expression analysis revealed Deg-CPR-2 expression in midguts and all the life-stages; however, there were differences in the expression levels across the life-stages. The enzyme activity of recombinant Deg-CPR-2 was inhibited in the presence of a cysteine protease inhibitor, which suggests that Deg-CPR-2 functions as a cysteine protease in PRMs. Finally, there was an in vitro increase in the mortality of PRMs, mainly protonymphs that were artificially fed with plasma from chickens immunized with Deg-CPR-2. These findings suggest that Deg-CPR-2 may contribute to protein digestion in the midgut of PRMs and is crucially involved in physiological processes in PRMs. Additionally, immunization with Deg-CPR-2 may reduce the number of protonymphs, and Deg-CPR-2 should be considered as a candidate antigen for anti-PRM vaccine development.

Key words: poultry red mite, cysteine protease, vaccine candidate, Deg-CPR-2, *Dermanyssus gallinae*

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INTRODUCTION

The poultry red mite (**PRM**), *Dermanyssus gallinae* (De Geer 1778), is a harmful hematophagous ectoparasite in chickens. It causes serious economic losses in the poultry industry, mainly in the laying hen sector (Sparagano et al., 2014). PRMs have 5 life-stages (eggs, larvae, protonymphs, deutonymphs, and adults) and start blood-sucking beginning from protonymphs (Sparagano et al., 2014). Protonymphs and deutonymphs moult after blood-sucking and proceed to the next life stage. Adult PRMs suck blood several times and oviposit eggs when feeding blood. PRM infestation in chicken causes anemia, reduced egg production and egg quality, decreased vaccination efficacy, abnormal behaviors such as feather pecking and cannibalism, and a low feed conversion ratio (Sparagano et al., 2014; Tomley and Sparagano, 2018). Additionally, PRMs may act as vectors in avian pathogen transmission (Sparagano et al., 2014), with several pathogens being detected or isolated in PRMs (Sparagano et al., 2014). Furthermore, PRM infestation has been reported to cause dermatitis in humans (Abdigoudarzi et al., 2013;

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Cinotti et al., 2015; Kavallari et al., 2018; Navarrete-Dechent and Uribe, 2018); moreover, zoonotic agents have been detected in PRMs (Raele et al., 2018). Therefore, PRMs may cause harm to human health and are a threat to the poultry industry.

Currently, pesticides are used for PRM eradication; however, PRMs have a characteristic of hiding in cracks and crevices of the poultry houses after sucking blood (Sparagano et al., 2014). Therefore, it is difficult to reach them with pesticides, which impedes PRM eradication efforts. Moreover, repeated and insufficient pesticide use selects for pesticide resistant PRMs in each farm (Sparagano et al., 2014). Consequently, it is difficult to control the number of PRMs in contaminated farms; therefore, other management strategies are required to ensure animal welfare and reduce economic losses in poultry farming (Sparagano et al., 2014). Alternative strategies for controlling PRMs have been studied and proposed, including developing repellents using plant oils (Lesna et al., 2012; Sparagano et al., 2013; Tabari et al., 2017; Camarda et al., 2018), applying pathogens or predators of PRMs (Lesna et al., 2012; Tomer et al., 2018), and vaccination of chicken to confer protective effects against PRMs (Bartley et al., 2009,2012,2015; Wright et al., 2009,2016; Lima-Barbero et al., 2019a,b; Tatham et al., 2019; Xu et al., 2020; Murata et al., 2021). Among these alternative strategies, there has been a focus on vaccination as a protective strategy since it could have prolonged effects if the antibody titre is adequately induced and the influence of environmental factors is reduced.

Several molecules have currently been reported as effective vaccine antigens against PRMs, including PRM proteins fractionated by detergents and urea (Wright et al., 2009); histamine release factor (Bartley et al., 2009); Cathepsin D and L-like proteinases (Bartley et al., 2012; Tatham et al., 2019); serine protease inhibitor, vitellogenin, hemelipoglycoprotein, and a protein of unknown function (Bartley et al., 2015); paramyosin and tropomyosin (Wright et al., 2016); akirin (Lima-Barbero et al., 2019a); calumenin (Lima-Barbero et al., 2019b); legumain (Xu et al., 2020); and cysteine protease (Murata et al., 2021). Among these candidates, proteases are among the suitable antigens for developing vaccines against PRMs since they are crucially involved in homeostasis, including anticoagulation for facilitating blood sucking and blood meal digestion. Cysteine proteases are crucial proteolytic enzymes involved in fundamental biological processes in various organisms, including catabolism and protein processing. Several cysteine proteases in some parasites have been characterized. Cysteine proteases in *Plasmodium falciparum* are involved in hemoglobin degradation, parasite egress, and surface protein processing (Verma et al., 2016). Moreover, cysteine protease expressed in the gut of flatworms and nematodes putatively contribute to host protein degradation (Caffrey et al., 2018). In ticks, cysteine proteases, including cathepsins B, C, and L (Sojka et al., 2013), are considered to be involved in hemoglobin digestion and are regarded as antigen candidates for antitick vaccines (Horn et al., 2009; Saidi et al., 2016). In PRMs, 2 cysteine proteases, *Dermanyssus gallinae* Cathepsin L-1 (**Dg-CatL-1**) (Bartley et al., 2012) and *Dermanyssus gallinae* Cysteine protease-1 (***Deg-CPR-1***) (Bartley et al., 2015; Murata et al., 2021), have been identified. Additionally, their use as vaccine antigens could affect the mortality and/or reproductive capacity of PRMs (Bartley et al., 2012, 2015; Xu et al., 2020; Murata et al., 2021).

We previously performed RNA-Seq analysis of starved and blood-fed PRMs to investigate gene expression profiles in PRMs (Fujisawa et al., 2020). Consequently, we identified a novel cysteine protease, which had higher expression intensity than did *Dg-CatL-1* (Bartley et al., 2012) and *Deg-CPR-1* (Bartley et al., 2015; Murata et al., 2021). As aforementioned, cysteine proteases are candidate vaccine antigens for the development of anti-PRM vaccines. Therefore, this study aimed to evaluate the potential of a novel cysteine protease, *Dermanyssus gallinae* cysteine protease-2 (**Deg-CPR-2**), as a vaccine antigen. Specifically, we aimed to investigate its genetic characteristics, enzyme activity, and gene expression, as well as to assess its efficacy as a vaccine antigen in vitro.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were approved by the Institutional Animal Care and Use Committee, Hokkaido University (Approval number: 20−0051). Moreover, all experiments were performed in accordance with the relevant guidelines and regulations of the Faculty of Veterinary Medicine, Hokkaido University, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Sample Collection

PRMs were collected into 50 mL bioreactor tubes with vent caps (Corning, NY) in a PRM-contaminated farm in Japan and transferred to the laboratory at 4°C. Some dark red and round PRMs, which were considered as blood-fed PRMs, were collected and placed in 70% ethanol within 48 h. Moreover, some of the PRMs in mixed stages were placed in 70% ethanol to collect eggs and larvae. The remaining PRMs were maintained in an incubator at 25°C for 1 wk and designated as starved PRMs. Some of the starved PRMs were placed in 70% ethanol while the remaining ones were maintained at an incubator at 5°C until use for in vitro feeding assays. Based on the size and morphology, PRMs fixed with 70% ethanol were sorted according to their life stages (eggs, larvae, protonymphs, deutonymphs, and adults) under a stereomicroscope SZX10 (Olympus, Tokyo, Japan). The sorted PRM samples were used for expression analysis.

RNA Preparation and cDNA Synthesis

The total RNA from each PRM sample was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. After treatment with DNase I (Invitrogen, Carlsbad, CA), complementary DNA (**cDNA**) was synthesized from 1 μg of the total RNA with PrimeScript Reverse Transcriptase (Takara Bio Inc., Shiga, Japan) using 200 pmol of oligo (dT)18 primer or 300 pmol of random hexamer primer (Hokkaido System Science, Hokkaido, Japan).

Determination of the Nucleotide Sequence of the Deg-CPR-2 Open Reading Frame

We previously analyzed the transcriptome of blood-fed and starved PRMs using RNA-Seq (Fujisawa et al., 2020). *Deg-CPR-2* was identified in the contigs yielded by RNA-Seq analysis data (BioSample accessions: SAMD00228960, SAMD00229086), which contained the open reading frame (**ORF**) sequence. To determine the nucleotide sequence, *Deg-CPR-2* ORF was amplified using Ex-Taq polymerase (TaKaRa Bio Inc.), with the sequence being verified using the GenomeLab GeXP Genetic Analysis System (Beckman Coulter Inc., Brea, CA).

Phylogenetic Analysis

To genetically characterize *Deg-CPR-2*, we performed phylogenetic analysis using cysteine protease genes previously reported in PRMs and other arthropods, including mites and ticks (Supplementary Table 1). The sequences were aligned using MEGA X software (Kumar et al., 2018). Subsequently, a maximum-likelihood phylogenetic tree was constructed using the same software with 1,000 bootstrap replicates. Moreover, a General Time Reversible model (Nei and Kumar, 2000) using discrete Gamma distribution (+G) was applied to improve the tree topology.

Laser-Capture Microdissection and cDNA Synthesis

For laser-capture microdissection (**LCM**), starved PRMs were fixed with 4% paraformaldehyde and embedded in paraffin. LCM was performed as previously reported (Ichii et al., 2017). Initially, 5-μm-thick paraffin sections were mounted on glass slides precoated with LCM films (Meiwafosis, Tokyo, Japan), deparaffinized with xylene, and dehydrated with alcohol. After staining using 1% toluidine blue for 5 s, we performed LCM of the salivary gland, midgut, and ovary using the MicroBeam Rel.4.2 (Carl Zeiss, Oberkochen, Germany), following the manufacturer's instructions. All procedures were performed in RNase-free conditions. Total RNA was purified using the RNAqueous Micro Total RNA Isolation kit (Thermo Fisher Scientific, Waltham, MA) and reverse transcribed to cDNA using the SuperScript First-Strand Synthesis System for reverse transcription polymerase chain reaction (**RT-PCR**) (Thermo Fisher Scientific), following the manufacturer's protocol.

Expression Analysis of Deg-CPR-2

Deg-CPR-2 messenger RNA (**mRNA**) expression in the samples from the salivary gland, midgut, and ovary, which were collected through LCM, was analyzed using standard PCR protocols with Ex-Taq polymerase (Takara Bio Inc.), following the manufacturer's protocol. *Deg-CPR-2* mRNA was detected using 2 primer sets targeting different regions. Moreover, we detected the *actin* gene as an internal control. As a negative control, distilled water was used instead of primers for PCR amplification. *Deg-CPR-2* mRNA expression in the different life stages, as well as in the fed and starved PRMs, was analyzed through PCR using Ex-Taq polymerase (Takara Bio Inc.). Supplementary Table 2 shows all the primers used for expression analysis. The amplified products were subjected to electrophoresis on a 2.0% agarose gel.

Regarding *Deg-CPR-2* mRNA expression in the different life-stages, we analyzed gene expression levels using quantitative PCR (**qPCR**). Using cDNA samples obtained from larvae, protonymphs, deutonymphs, and adults in each feeding state, qPCR was performed with LightCycler480 System II (Roche Diagnostics, Mannheim, Germany) using TB Green Premix DimerEraser (TaKaRa Bio Inc.), following the manufacturer's instructions. The *elongation factor 1-alpha-like* (***Elf1a-like***) gene was amplified as an internal control (Ariizumi et al., 2021). Supplementary Table 2 shows the primers used for qPCR. The cycling conditions consisted of initial denaturation at 95°C for 30 s, followed by 45 cycles at 95°C for 5 s, 53°C for 30 s, and 72°C for 30 s. To evaluate the specificity of primer pairs, we performed a final melting curve analysis from 65°C to 95°C at a rate of 0.1°C/s. To generate standard curves for quantification, we used serial dilutions of T-vector pMD20 (TaKaRa Bio Inc.) inserted with *Deg-CPR-2* or *Elf1a1*. *Deg-CPR-2* mRNA expression was presented as the ratio obtained by dividing the mRNA concentrations of *Dg-Ctr1* and *Elf1a1*.

Expression of Recombinant Proteins

The recombinant Deg-CPR-2 protein was expressed as a fusion protein with His-tag using the BIC system (Takara Bio Inc.). The *Deg-CPR-2* ORF without the signal peptide was amplified with KOD-Plus-Neo (TOYOBO Co., Ltd., Osaka, Japan) using specific primers containing homologous recombination sites for introduction into the pBIC3 DNA vector (Takara Bio Inc.). Amplified fragments were introduced into *Brevibacillus* Competent Cells (Takara Bio Inc.), followed by the introduction into pBIC3 DNA vector through homologous recombination. The transformed bacteria were cultured in 10 mL of Thayer−Martin (**TM**) medium for 16 h at 25°C. After precultivation, 90 mL of

TM medium was added to the culture, followed by culturing of bacteria for 24 h at 25°C. The recombinant proteins were purified using Ni Sepharose Fast Flow (GE Healthcare, Chicago, IL) and eluted with 50 mM Tris-HCl, 500 mM NaCl, and 250 mM imidazole (pH 7.5). Subsequently, the eluted fractions were dialysed with phosphate-buffered saline (**PBS**) using SnakeSkin Dialysis Tubing, 10 K MWCO (Thermo Fisher Scientific) overnight at 4°C. The protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), following the manufacturer's protocols. To confirm protein purification, the obtained proteins were lysed in 2 × sodium dodecyl sulphate (**SDS**) buffer (125 mM Tris−HCl [pH 6.8], 4% SDS, 10% 2-mercaptoethanol, and 20% glycerol), boiled for 5 min, separated using 10% SDS-polyacrylamide gel electrophoresis (**SDS-PAGE**), and stained with Coomassie brilliant blue (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Immunisation With Deg-CPR-2

Three 3-wk-old chickens (Hy-Line Brown) were subcutaneously immunized with 20 μg of Deg-CPR-2. The recombinant Deg-CPR-2 was diluted in PBS and mixed with light liquid paraffin as the adjuvant. After 4 wk, 20 μg of Deg-CPR-2 with the same adjuvant was used for a second immunization round. As a control, 3 chickens were subcutaneously injected with PBS mixed with light liquid paraffin at 3 and 7 wk old. Plasma was isolated from the heparinized blood of the immunized and control chickens at 10 wk old. The obtained plasma was used for the in vitro feeding assay. All chickens were raised in the animal facility at the faculty of Veterinary Medicine, Hokkaido University.

Western Blotting

Western blotting was used to examine the production of specific antibodies against Deg-CPR-2. Purified Deg-CPR-2 was separated using a 10% SDS-polyacrylamide gel; subsequently, it was transferred onto polyvinylidene difluoride membranes (Merck Millipore, Burlington, MA). The membranes were blocked overnight with 0.05% Tween 20 in PBS (**PBST**) that contained 1% skim milk at 4°C. The membranes were incubated at 25°C with plasma obtained from immunized chickens, washed thrice using PBST, and incubated at 25°C with an anti-chicken IgY peroxidase rabbit antibody (Merck Millipore). Finally, the membranes were incubated with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) to visualize the peroxidase signal.

Enzymatic Activity of the Deg-CPR-2 Protein

We performed enzyme activity assays for Deg-CPR-2 using two substrates from SensoLyte Rh110 Cathepsin L Assay Kit (AnaSpec, Inc., Fremont, CA) and Cathepsin D Assay Kit, Fluorometric, SensoLyte (AnaSpec, Inc.), following the manufacturer's instructions. Moreover, a cysteine protease inhibitor (CPR inhibitor), which is a component of the SensoLyte Rh110 Cathepsin L Assay Kit (AnaSpec), was used to characterize the cysteine protease function of Deg-CPR-2. Furthermore, an aspartic protease inhibitor (ASP inhibitor; pepstatin A), which is a component of Cathepsin D Assay Kit, Fluorometric, SensoLyte (AnaSpec, Inc.), was used as a control in the assays. Fluorescence was detected at excitation/emission wavelengths of 490/530 nm. Enzyme activity was assessed using 1.0, 2.0, and 4.0 μg of recombinant proteins.

Enzyme-Linked Immunosorbent Assay

Antibody titres in the immune plasmas were determined through enzyme-linked immunosorbent assay (**ELISA**). The recombinant Deg-CPR-2 was coated on the wells of 96-well plates (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) (100 ng/well) at 4°C overnight with carbon-bicarbonate buffer (pH 9.8). After removing the recombinant protein, PBST-containing 1 % bovine serum albumin was added into each well. Next, the plates were incubated at 37°C for 2 h. After blocking, immune plasma diluted 8,000-, 16,000-, and 32,000-fold was added into each well; subsequently, the plates were incubated at 25°C for 30 min. After incubation, the wells were washed 5 times with PBST and incubated at 37°C with anti-chicken IgY[IgG](H+L)-HRP, Goat (Bethyl laboratories, Montgomery, TX) for 1 h. As a substrate, TMB One Component HRP Microwell Substrate (Bethyl Laboratories, Inc.) was added into each well, followed by incubation at 37°C for 15 min. After adding 100 μL of 0.18 M H_2SO_4 into each well, the sample absorbance was measured at 450 nm. Plasma from control chickens was diluted 2,000-fold, with its absorbance being measured as described. The cut-off value was set at optical density $(\mathbf{OD})_{450} = 0.13$; further, the antibody titre was indicated as the maximum dilution rate.

In Vitro Feeding Assay

An in vitro feeding assay was performed as previously described (Ariizumi et al., 2021). Plasma obtained from fresh and heparinised chicken blood was replaced with that obtained from immunized or control chicken blood. Fresh blood was obtained from chickens raised at the Field Science Center for Northern Biosphere, Hokkaido University. Blood feeding was performed at 40°C for 4 h; subsequently, approximately 20 blood-feeding PRMs were collected using Pasteur pipettes. The mortality of blood-feeding PRMs was monitored daily for 1 wk. The in vitro feeding assays were performed three times (experiments 1, 2 and 3). The survival rate of the PRMs was monitored for 7 d after being fed immune or control plasma. In experiment 1, the survival rate of PRMs in varying life stages was assessed. In experiment 2, the survival rate of PRMs in varying life stages was assessed. In addition, the mortality of adults and nymphs was

analyzed separately. In experiment 3, the survival rate of adults and protonymphs was analyzed separately. The adults and protonymphs could be distinguished morphologically under the microscope. The total number of blood-feeding PRMs monitored in each experiment was as follows: experiment 1: Deg-CPR-2, $n = 114$; control, $n = 130$, experiment 2: Deg-CPR-2, $n = 159$ (adults, $n = 67$; nymphs, $n = 92$); control, $n = 95$ (adults, $n = 55$; nymphs, $n = 40$), and experiment 3: Deg-CPR2: adults, $n = 125$; protonymphs, $n = 83$; control: adults, $n = 120$; protonymphs, $n = 84$. The anti-PRM effect was assessed based on the mortality in all 3 experiments. In experiment 2, most of the nymphs were protonymphs, but a few deutonymphs were included. In addition, in experiment 2 the reproductive capacity (fecundity) and population growth rate were assessed. The population growth rate and reproductive capacity were estimated as follows:

1) (Population growth rate) = (Total number of PRMs alive on d 7) / (Total number of blood-fed PRMs on d 0)
   - The number of PRMs alive on d 7 included newborn PRMs (larvae and protonymphs).
2) (Reproductive capacity) = [Total number of newborn PRMs (larvae and protonymphs) on d 7) / (Total number of adults fed on blood on d 0)
   - The numerator included newly-born protonymphs given that larvae develop protonymphs within a few days without blood-feeding.

Statistical Analysis

To compare PRM mortality between the immunized and control groups after in vitro feeding, we generated Kaplan−Meier curves and performed a log-rank test. Additionally, we performed between-group comparisons of the mortality of PRMs on each day using Fisher's exact test. Moreover, the odds ratio and 95% confidence interval (**CI**) were estimated. In the enzyme activity assays, all values are reported as mean ± standard deviation. Further, a statistical comparison was performed using the one-way analysis of variance, with Tukey's HSD test for post hoc comparisons. All statistical analyses were performed using EZR (Kanda, 2013). Statistical significance was set at $P < 0.05$.

RESULTS

Determination and Expression Intensities of Deg-CPR-2

We previously performed RNA-Seq analysis to analyze gene expression in blood-fed and starved PRMs (BioSample accessions: SAMD00228960, SAMD00229086); further, we searched the domains and motifs using the InterProScan program v5.32-71.0 (http://bioinf.wehi.edu.au/edgeR/) (Fujisawa et al., 2020). Among the detected contigs, one contained a C1A peptidase domain, which suggests that the gene encodes a cysteine protease-like protein. The contig contained the ORF of the cysteine protease-like gene; accordingly, we named the cysteine protease-like protein Deg-CPR-2. We cloned the ORF of *Deg-CPR-2* and verified its nucleotide sequence through Sanger sequencing (data not shown). Based on the BLAST search, Deg-CPR-2 was predicted as a cysteine protease with a propeptide inhibitor domain (prodomain) at positions 27−86 and a peptidase C1A subfamily domain (mature domain) at positions 115−329. Moreover, four amino acid residues were predicted as active sites for peptidase (Figure 1A). Additionally, ERFNIN motif- and GNFD motif-like sequences, which are conserved in the prodomain of cathepsin L proteases (Verma et al., 2016), were identified in the Deg-CPR-2 prodomain (Figure 1A). The sequence of the ERFNIN motif-like in the Deg-CPR-2 prodomain was VRFNIN; further, Deg-CPR-2 has an amino acid difference at position 43. Regarding the GNFD motif, the Deg-CPR-2 sequence was RNYD; moreover, 2 amino acid differences were observed at positions 75 and 79. Analysis of the expression intensity (Fujisawa et al., 2020) revealed that it was higher in *Deg-CPR-2* than in other previously reported cysteine proteases, *Deg-CPR-1* and *Dg-CatL-1*, for both starved and blood-fed PRMs (Supplementary Table 3).

Phylogenetic Analysis of Deg-CPR-2

To investigate the genetic characteristics of *Deg-CPR-2*, we performed phylogenetic tree analysis using cysteine proteases from other arthropods, including other mite species and ticks. As previously reported, the cysteine proteases were separated into three clusters (Figure 1B; Murata et al., 2021). The *Deg-CPR-2* genes belonged to cluster 1; moreover, they were closely associated with the cysteine proteases from other mite species, including predatory and varroa mites. Cluster 1 also included cathepsin L-like proteases from ticks. Among the cysteine proteases classified into cluster 1, some were considered necessary for hemoglobin digestion (Sojka et al., 2013). Other cysteine proteases, *Deg-CPR-1* and *Dg-CatL-1*, which have been previously reported in PRMs, were classified into clusters 2 and 3, respectively. Upon alignment of the deduced amino acid sequence of Deg-CPR2 with those of cysteine proteases identified in ticks that were involved in blood digestion, the mature domain was highly conserved and 4 active sites for the peptidase were completely conserved (Figure 1A). Therefore, the phylogeny of *Deg-CPR-2* appears to be closely similar to that of cysteine proteases identified in ticks as being associated with blood digestion; moreover, it clearly differed from that of *Deg-CPR-1* and *Dg-CatL-1*.

Deg-CPR-2 Expression Analysis

To further characterize Deg-CPR-2, we performed *Deg-CPR-2* mRNA expression analysis. First, we

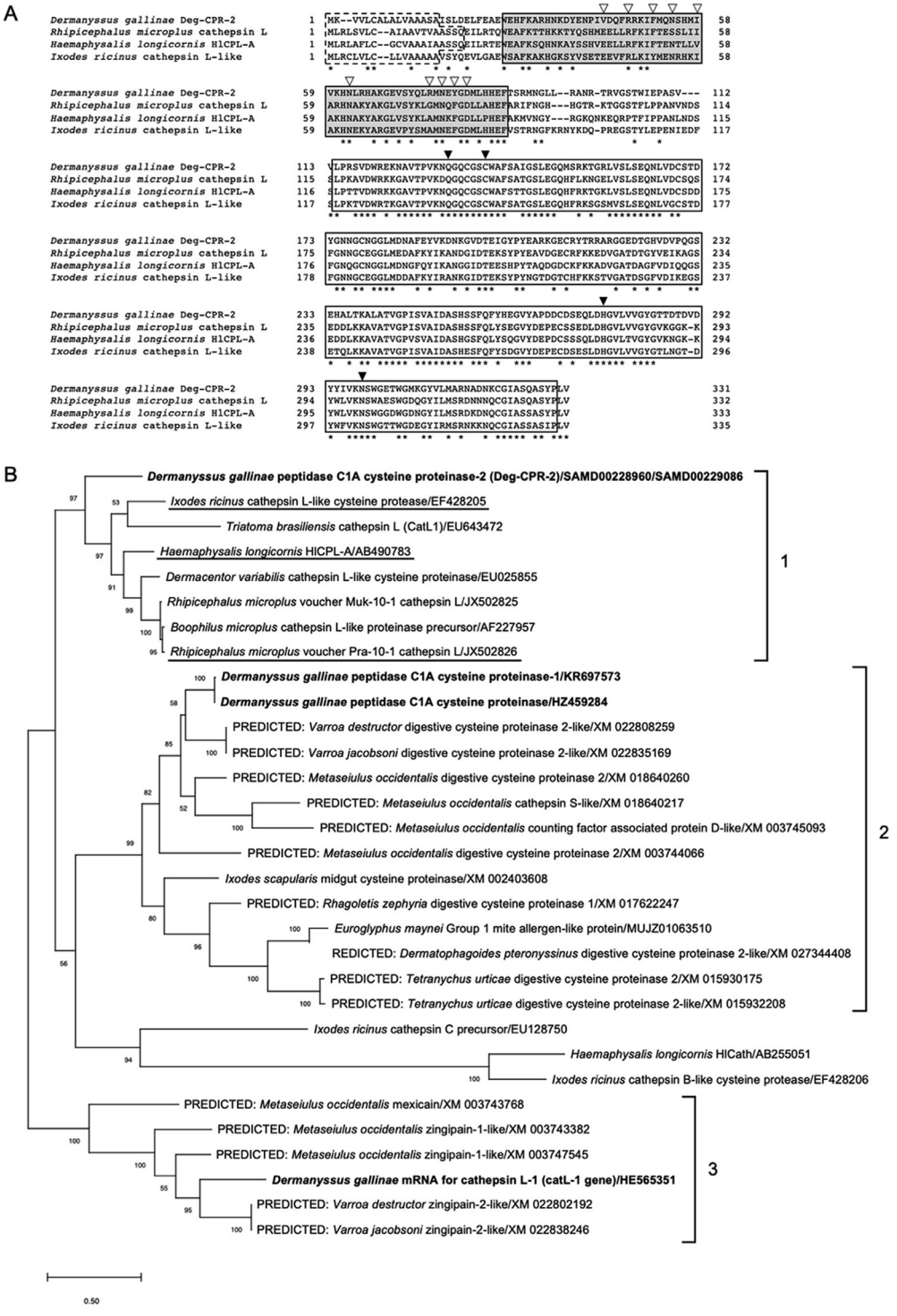

Figure 1. Genetic characterization of *Dermanyssus gallinae* cysteine protease-2 (Deg-CPR-2). (A) The deduced amino acid sequence of Deg-CPR-2 was aligned to that of cysteine proteases previously proposed to be involved in haemoglobin digestion in ticks. The dotted-lined, gray-shaded, and solid-lined boxes indicate the signal peptide sequences, propeptide inhibitor domain (prodomain), and peptidase C1A subfamily domain (mature domain), respectively. The white arrows indicate the ERFNIN and GNFD motifs conserved in the prodomain of cathepsin L proteases. The black arrows indicate the active sites for peptidase activity. (B) Phylogenetic tree based on the nucleotide sequences of the open reading frames of the *cysteine protease* genes in poultry red mites (PRMs) and other arthropods, including other mites and ticks. The tree was constructed using the maximum-likelihood method with MEGA X software (Kumar et al., 2018). The numbers on the right indicate the clusters. Cluster 1 included cathepsin L-like proteases, which are thought to be involved in haemoglobin digestion in ticks (underlined), and a cysteine protease from PRMs (*Deg-CPR-2*) (bold font). The other PRM cysteine proteases, *Deg-CPR-1* and *Dg-CatL-1*, belonged to clusters 2 and 3, respectively (bold font).

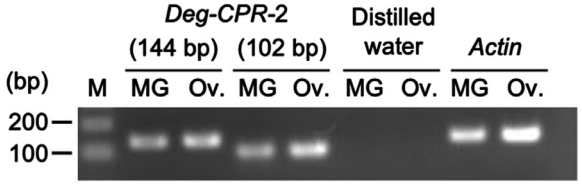

Figure 2. Expression analysis of *Deg-CPR-2* mRNA in the midguts and ovaries of PRMs. The tissue samples were collected from starved deutonymphs and adults through laser-capture microdissection; moreover, RT-PCR was performed to detect *Deg-CPR-2* mRNA expression in the midgut and ovary. *Deg-CPR-2* was detected using two different sets of primer pairs (product size: 144 bp and 102 bp). The *actin* gene was amplified as an internal control in all expression analyses. As a negative control, distilled water was used in place of primers to detect *Deg-CPR-2* mRNA. Abbreviations: MG, midgut; Ov, ovary.

analyzed *Deg-CPR-2* expression in the midguts through RT-PCR analysis using the LCM samples. We used 2 sets of primer pairs to amplify the *Deg-CPR-2* gene, which both detected the *Deg-CPR-2* gene in the midguts (Figure 2). Additionally, *Deg-CPR-2* mRNA was observed in ovaries. Next, we investigated *Deg-CPR-2* gene expression in each life stage of the starved and blood-fed PRMs. *Deg-CPR-2* expression was observed in PRM samples from all the life-stages, regardless of blood-feeding (Figure 3A). Additionally, *Deg-CPR-2* expression was quantified in each life stage, except for eggs, using qPCR. There was higher *Deg-CPR-2* expression in the starved deutonymphs and both feeding states for adults; however, *Deg-CPR-2* expression was observed in all analyzed stages (Figure 3B). These results suggest that Deg-CPR-1 is an antigen that might be constitutively expressed in midguts, regardless of blood feeding; however, the expression level appeared to differ across the blood-fed states and life-stages.

Expression of the Recombinant Deg-CPR-2 Protein and Its Enzyme Activity

We expressed Deg-CPR-2 without signal peptides (Figure 1B) as recombinant proteins fused with His-tag. For recombinant protein expression, we used *Brevibacillus choshinensis*, which are gram-positive bacteria that secreted the target recombinant proteins into the cultured medium. Recombinant Deg-CPR-2 expression in the supernatants and cells was confirmed using western blotting; moreover, we confirmed Deg-CPR-2 secretion into the cultured medium (Figure 4A). Subsequently, we purified the recombinant protein through affinity chromatography, with the purity being confirmed using SDS-PAGE and western blotting (Figures 4B and 4C).

To investigate the protease functions of Deg-CPR-2, we performed an enzyme activity assay using fluorescent

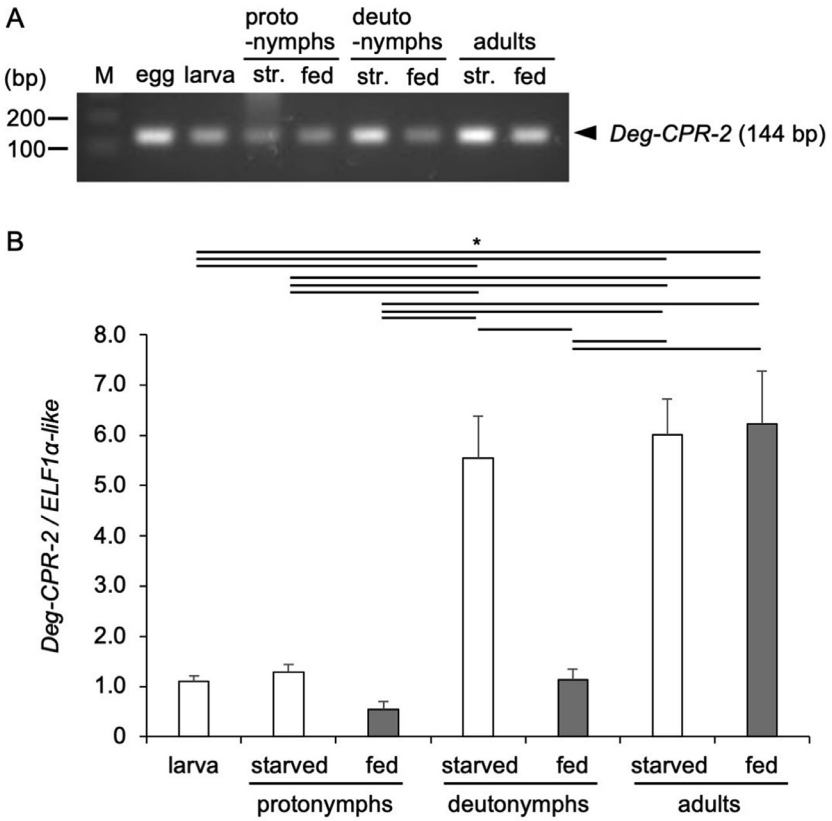

Figure 3. Expression analysis of *Deg-CPR-2* mRNA in different life-stages. Based on their morphology and body size, a portion of the starved and blood-fed PRMs were sorted based on their life stage (eggs, larvae, protonymphs, deutonymphs, and adults) under a stereomicroscope; moreover, *Deg-CPR-2* mRNA was detected using RT-PCR (A). *Deg-CPR-2* mRNA expression was quantified using qPCR in life stages, except for eggs (B). The *elongation factor 1-alpha-like* (*Elf1a-like*) gene was amplified as the internal control; furthermore, *Deg-CPR-2* mRNA expression was presented as the ratio obtained by dividing the concentrations of *Deg-CPR-2* and *Elf1a1* mRNA. Abbreviations: Ad, adult; DN, deutonymph; NC, negative control (distilled water); PC, positive control (cDNA from PRMs of all stages); PN, protonymph.

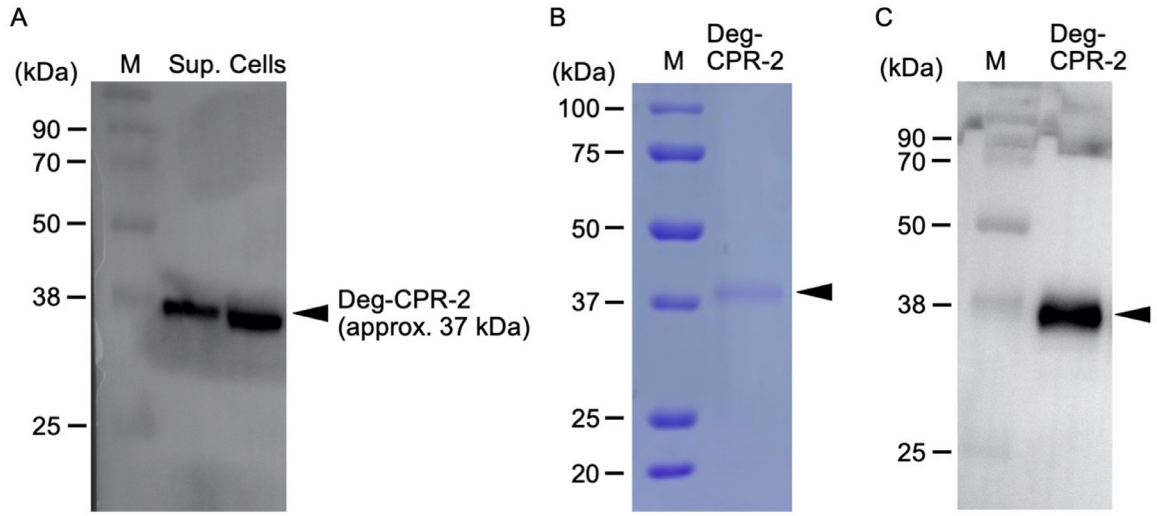

Figure 4. Expression and purification of the recombinant Deg-CPR-2 protein. We expressed and purified the entire recombinant Deg-CPR-2 without the signal peptides and fused with the histidine tag. The recombinant Deg-CPR-1 was expressed using the *Brevibacillus* expression system; moreover, it was purified using metal affinity resins from the cultured supernatants. Recombinant Deg-CPR-2 expression in the supernatants and bacterial cells was confirmed through western blotting (A). The purity of the purified protein was confirmed through SDS-PAGE (B) and western blotting (C). M, Marker (Precision Plus Protein All Blue Prestained Protein Standards, Bio-Rad, CA).

substrates from 2 commercial kits. First, we found that Deg-CPR-2 had dose-dependent cathepsin L activity, which was significantly reduced by adding a CPR inhibitor (Figure 5A). Further, we assessed enzymatic activity using cathepsin D, which is an aspartic protease. Notably, Deg-CPR-2 indicated the enzymatic activity, which was inhibited by the presence of a CPR inhibitor but not an ASP inhibitor, pepstatin A (Figure 5B). These results suggest that Deg-CPR-2 has cysteine protease activity.

Assessment of the Acaricidal Activity of Plasma Obtained From Chickens Immunised With Deg-CPR-2

Finally, we evaluated the potential of Deg-CPR-2 as a vaccine antigen in vitro based on the mortality of PRMs fed with immune plasma (Murata et al., 2021), with the in vitro feeding being performed, as previously reported (Ariizumi et al., 2021). The experiments were performed three times (experiments 1, 2, and 3). For immune plasma preparations, the chickens were immunized with recombinant Deg-CPR-2. The production of antibodies specific to Deg-CPR-2 in the immune plasma was confirmed by western blotting (Figure 6A). Additionally, we analyzed the antibody titre in the immune plasma, which was increased in all the chickens (Table 1).

After in vit*ro* feeding, the mortality of PRMs fed with fresh blood, whose plasma was replaced with immune or control plasma, was monitored for 7 d. To evaluate the acaricidal potential of Deg-CPR-2 immunization, we performed between-group comparisons of the mortality rate. In experiment 1, the mortality of PRMs fed with control plasma increased on d 5 and 7 after feeding (Table 2). Moreover, the odds ratio revealed increased mortality of PRMs on d 5 and 7 after feeding. Comparison of the Kaplan−Meier curves revealed a significant increase in the mortality of PRMs fed with immune plasma, relative to that of PRMs fed with control plasma (Figure 6B). Therefore, we confirmed the potential of anti-PRM effects by immunization with Deg-CPR-2.

However, in experiment 2, there was no significant between-group difference in mortality at all time-points (Table 3, Figure 7A). In experiments 1 and 2, we used PRMs at mixed stages for the assays. Unfortunately, we lacked data regarding the PRM composition in experiment 1. However, we described the composition of PRMs (adults or [proto]nymphs) in experiment 2 to analyze the reproduction capacity. Therefore, we separately analyzed the mortality of adults and nymphs. In this assay, nymphs were mainly comprised of protonymphs; however, a few deutonymphs were included. Fisher's exact test revealed no significant difference in the mortality at all time-points between adults and nymphs; however, there was a tendency of higher mortality among nymphs than among adults (Supplementary Tables 4 and 5). Although the log-rank test also did not show a significant difference in mortality between nymphs and adults, mortality tended to be higher among nymphs than among adults (Figures 7B and 7C). Further, in experiment 2, we analyzed the reproduction capacity of adults and the population growth rate of PRMs at mixed stages at 7 post-feeding days. Although there was a tendency for a reduction in both parameters in PRMs fed with immune plasma, there were no significant differences (Supplementary Tables 6 and 7).

We further assessed the acaricidal effects of the immune plasma on protonymphs to eliminate the bias due to the differences in the number of protonymphs in experiment 2. In experiment 3, we monitored the

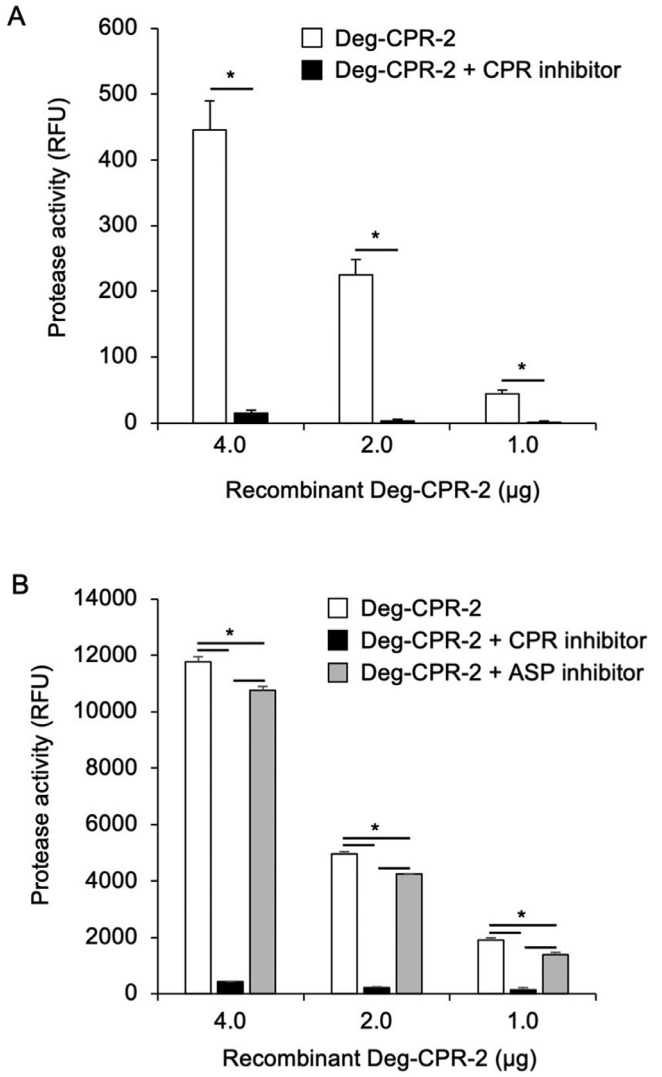

Figure 5. Enzyme activity of Deg-CPR-2. The enzyme activity of Deg-CPR-2 was assessed using fluorescent substrates from commercial kits. In addition, enzyme activity was assessed in the presence of a cysteine protease inhibitor or an aspartic inhibitor (pepstatin A). The x-axis shows the amounts of recombinant proteins used in each assay. Protease activity was indicated as relative fluorescence units (RFU). Error bars indicate standard deviations. *Results with $P < 0.05$ were considered significant.

survival rate of adults and protonymphs separately. The mortality of adults fed with immune plasma was significantly higher on d 7 after feeding than that of adults fed with control plasma (Table 4), and comparison of the Kaplan−Meier curves revealed a significantly higher mortality in PRMs fed with immune plasma than in PRMs fed with control plasma (Figure 8A). In contrast, the mortality of protonymphs fed with immune plasma was higher than those of control on d 3, 4, 5 after feeding but there was no significant between-group difference in mortality on d 6 and 7 after being fed with plasma (Table 5 and Figure 8A). The mortality of protonymphs in the control group rapidly increased on d 6 and 7 after feeding (40 and 69%, respectively), possibly due to the conditions under which they were kept in this experiment. In contrast to experiment 3, in experiments 1 and 2, the cumulative mortality in the control group was much higher than that in the control group on d 7. However, in experiment 3, the increase in mortality of the protonymphs fed with immune plasma was occurred earlier than that in the control group (Table 5). Considered together, these experiments suggest that immune plasma from chickens immunized with Deg-CPR-2 may have had acaricidal effects on protonymphs but that its effect on adult PRMs is weak.

DISCUSSION

This study investigated the characteristics of a cysteine protease isolated from PRMs, Deg-CPR-2, which differed from other previously reported cysteine proteases, Deg-CPR-1 and Dg-CatL-1. Moreover, we assessed its potential as a vaccine antigen. In phylogenetic analysis, *Deg-CPR-2* was classified into a cluster different from those of the other cysteine proteases; moreover, this cluster included cathepsin L-like proteases predicted to be enzymes associated with hemoglobin digestion in the midguts of ticks (Sojka et al., 2013). Expression analysis revealed *Deg-CPR-2* expression in midguts; moreover, *Deg-CPR-2* expression was confirmed in all life stages. However, *Deg-CPR-2* expression levels differed across the life stage, with adults and starved deutonymphs showing a higher expression. The enzyme activity of the recombinant Deg-CPR-2 was inhibited in the presence of a cysteine protease, but not an aspartic protease, inhibitor. Moreover, a tendency of higher mortality among protonymphs fed with immune plasma was observed. Taken together, these findings suggest that Deg-CPR-2 has cysteine protease activity, which may contribute to protein digestion in the midgut of PRMs. Moreover, they suggest that immunization with this protein could control the PRM population by suppressing the growth of PRMs, mainly protonymphs.

The basic structure of the cathepsin L subfamily (cathepsins L, V, K, S, W, F, and H), which belongs to cysteine proteases, is comprised of a propeptide inhibitor domain (prodomain) and a peptidase C1A subfamily domain (mature domain). As shown in Figure 1A, the mature domain was highly conserved among those in arthropods. The characteristic motifs are highly conserved in the prodomain of the cathepsin L subfamily; moreover, the motifs are termed ERFNIN and GNFD based on their amino acid sequences. Contrastingly, other cathepsins B, C, O, and X, which are also cysteine proteases, lack the ERFNIN motif (Saidi et al., 2016). Among the cysteine proteases encoding both motifs, the amino acid residues of ERFNIN at the first, third, and fourth positions (E, F, and I) are often substituted by other residues (Pandey et al., 2009). Additionally, amino acid substitutions were observed at the G and F positions of the GNFD motifs in some cysteine proteases (Pandey et al., 2009). The sequences of ERFNIN and GNFD-motifs in the prodomain of Deg-CPR-2 were VRFNIN and RNYD, respectively, which differed from the Deg-CPR-1 and Dg-CatL-1 sequences (Deg-CPR-1: ERFNVN and KNRD, Dg-CatL-1: ERFKIN and RSYD). Therefore, amino acid substitutions in both

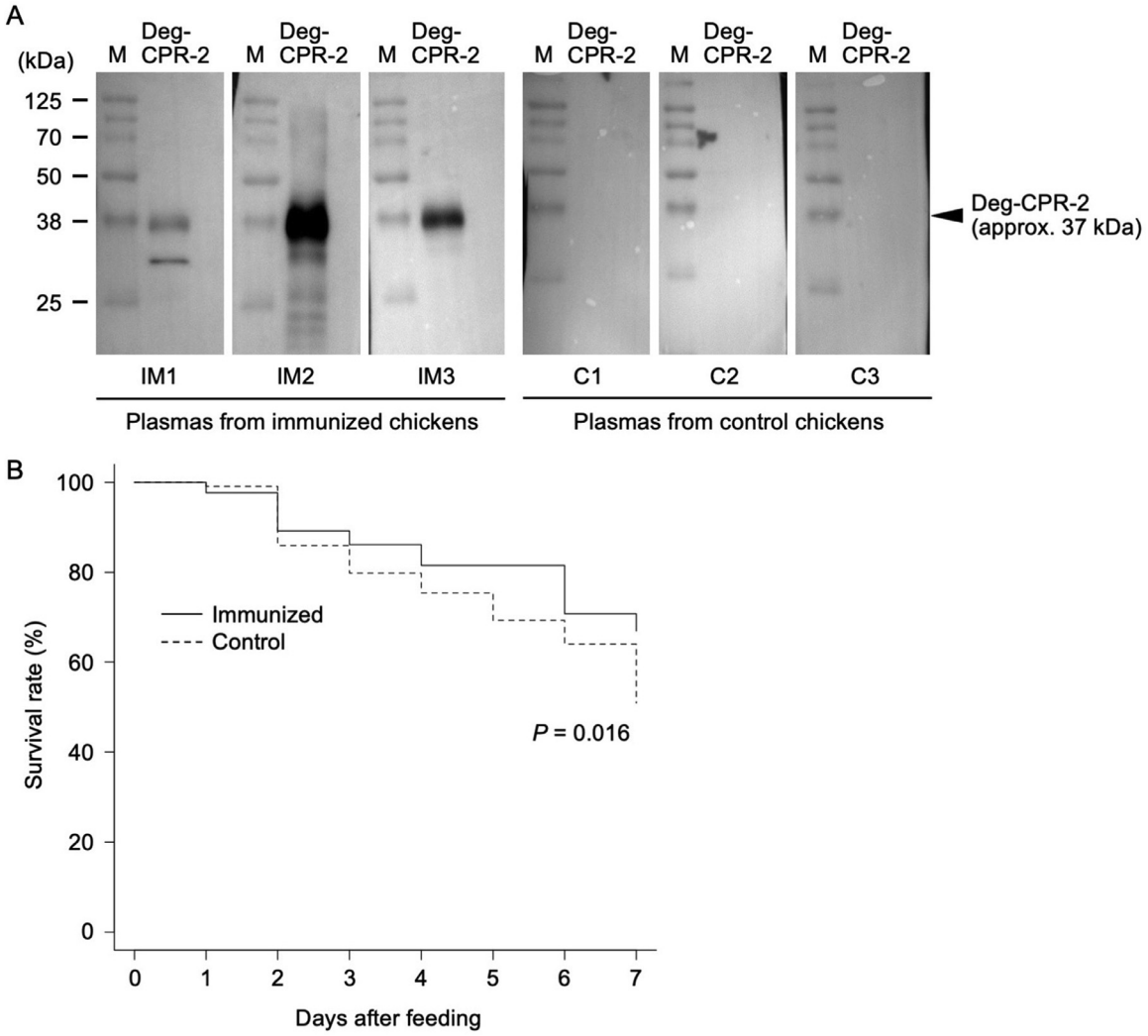

Figure 6. Assessment of the acaricidal potential of plasma obtained from chickens immunized with Deg-CPR-2. (A) Antibody production in the plasma from chickens immunized with Deg-CPR-2. Three chickens were immunized with Deg-CPR-2 while another three chickens were inoculated with PBS. Plasma was obtained from immunized and control chickens; subsequently, the plasma antibody specific to Deg-CPR-2 was detected through western blotting. The arrowhead indicates the predicted molecular weight of Deg-CPR-2 (37 kDa). (B) The survival rate of PRMs fed with immune and control plasma was assessed daily for 1 wk. The PRMs were artificially fed with plasma using in vitro feeding assays. The in vitro feeding assays were performed (experiment 1). The total number of PRMs fed with plasma was as follows: experiment 1: fed with immune plasma: $n = 114$, fed with control plasma: $n = 130$. The number of dead PRMs was recorded and plotted on the graph to generate Kaplan−Meier curves. Statistical analysis was performed using a log-rank test. *Results with $P < 0.05$ were considered statistically significant. Abbreviations: PBS, phosphate-buffered saline; PRM, poultry red mite.

motifs may not affect their roles. Taken together, Deg-CPR-2 appears to have similar characteristics to the cathepsin L subfamily.

The cathepsin L subfamily is expressed as zymogens containing the prodomain, which blocks the interaction of the mature domain with the substrates to prevent unnecessary protein degradation (Verma et al., 2016).

Table 1. Antibody titres in plasma samples from chickens immunized with recombinant Deg-CPR-2.

Group	Chicken	Antibody titre
Control	C1	< 2,000
	C2	< 2,000
	C3	< 2,000
Immunised	IM1	8,000
	IM2	> 32,000
	IM3	> 32,000

Generally, zymogen conversion from the inactive form is induced by the presence of other enzymes and accessory molecules, as well as auto-catalytic processes caused by a pH change (Verma et al., 2016). In this study, we expressed the entire Deg-CPR-2 region, except for the signal peptides, and assessed its enzymatic activity. Deg-CPR2 successfully degraded two substrates and showed enzymatic activity, which was inhibited by the presence of a cysteine protease inhibitor. Therefore, Deg-CPR-2 may be digested by an autocatalytic process depending on the pH conditions in the assays, and therefore might exhibit cysteine protease activity. Further research is required to clarify the biological functions, including the substrate specificity and involvement in hemoglobin digestion, of Deg-CPR-2 in PRMs; moreover, assessment using the mature Deg-CPR-2 form lacking the prodomain may be necessary. In addition, to

Table 2. Cumulative mortality of poultry red mites fed with plasma obtained from chickens immunized with Deg-CPR-2 (experiment 1).

	Days after feeding						
	1	2	3	4	5	6	7
PRMs fed immune plasma ($n = 114$)							
No. of dead PRMs post-feeding	1	16	23	28	35	41	56
Mortality (%)	0.88	14.04	20.18	24.56	30.70	35.96	49.12
PRMs fed control plasma ($n = 130$)							
No. of dead PRMs post-feeding	3	14	18	24	24	38	43
Mortality (%)	2.31	10.77	13.85	18.46	18.46	29.23	33.08
P value (Fisher's exact test)	0.625	0.443	0.230	0.274	0.035	0.276	0.01*
Odds ratio	0.38	1.35	1.57	1.44	1.95	1.36	1.95
95% CI	0.01−4.76	0.58−3.15	0.76−3.29	0.74−2.79	1.04−3.73	0.77−2.41	1.13−3.39

Abbreviations: CI, confidence interval; PRM, poultry red mite
*P values < 0.05 were considered statistically significant.

Table 3. Cumulative mortality of poultry red mites fed with plasma obtained from chickens immunized with Deg-CPR-2 (experiment 2).

	Days after feeding						
	1	2	3	4	5	6	7
PRMs fed immune plasma ($n = 159$)							
No. of dead PRMs post-feeding	3	9	10	21	35	57	70
Mortality (%)	1.89	5.66	6.29	13.21	22.01	35.85	44.03
PRMs fed control plasma ($n = 95$)							
No. of dead PRMs post-feeding	2	4	5	5	16	25	34
Mortality (%)	2.11	4.21	5.26	5.26	16.84	26.32	35.79
P value (Fisher's exact test)	1.000	0.772	1.000	0.053	0.337	0.129	0.24
Odds ratio	0.89	1.36	1.21	2.73	1.39	1.56	1.41
95% CI	0.10−10.90	0.37−6.24	0.36−4.65	0.96−9.60	0.70−2.88	0.87−2.87	0.81−2.47

Abbreviatioms: CI, confidence interval; PRM, poultry red mite.

Table 4. Cumulative mortality of adult poultry red mites fed with plasma obtained from chickens immunized with Deg-CPR-2 (experiment 3).

	Days after feeding						
	1	2	3	4	5	6	7
Adults fed immune plasma ($n = 125$)							
No. of dead adults	2	5	5	6	6	19	33
Mortality (%)	1.60	4.00	4.00	4.80	4.80	15.20	26.40
Adults fed control plasma ($n = 120$)							
No. of dead adults	1	2	2	2	3	12	17
Mortality (%)	0.83	1.67	1.67	1.67	2.50	10.00	14.17
P value (Fisher's exact test)	>0.999	0.447	0.447	0.282	0.500	0.252	0.027*
Odds ratio	1.930	2.450	2.450	2.963	1.961	1.610	2.098
95% CI	0.10−115.02	0.39−26.22	0.39−26.22	0.52−30.59	0.41−12.40	0.70−3.83	1.06−4.30

Abbreviation: CI, confidence interval.
*P values < 0.05 were considered statistically significant.

clarify the mechanisms of vaccine effects, it needs to be determined whether antibodies induced by immunization of chickens with Deg-CPR-2 can inhibit its protein functions, such as enzyme activity and hemoglobin digestion, using purified IgY.

In the expression analysis of each life stage, adults and starved deutonymphs showed higher *Deg-CPR-2* expression levels than did those in other life stages. There was a significant difference in the expression levels between starved and blood-fed deutonymphs. Contrastingly, adults revealed higher expression levels in both starved and blood-fed states. Deutonymphs moult and become adults within a few days of starting to feed on blood. On the other hand, adults repeatedly fed on blood several times for oviposition (Sparagano et al., 2014). Therefore, *Deg-CPR-2* expression in deutonymphs may be temporarily decreased after blood-sucking. On the other hand, higher *Deg-CPR-2* expression levels in adults may be maintained even after blood-sucking. To clarify *Deg-CPR-2* expression patterns, there is a need for more detailed profiling of temporal changes in the expression levels in each life stage.

Protonymphs showed lower *Deg-CPR-2* expression levels than did those in starved deutonymphs and adults. However, immune plasma showed higher acaricidal effects in nymphs, mainly protonymphs, which suggests that Deg-CPR-2 may play more vital physiological roles in protonymphs than in adults. Some cysteine proteases are encoded in various organisms (Verma et al., 2016); moreover, 2 other cysteine proteases, Dg-CatL-1 and Deg-CPR-1, have been identified in PRMs (Bartley et al., 2012, 2015; Murata et al., 2021). Therefore, in adults, other cysteine proteases may compensate for Deg-CPR-2 functions blocked by immune plasma. In

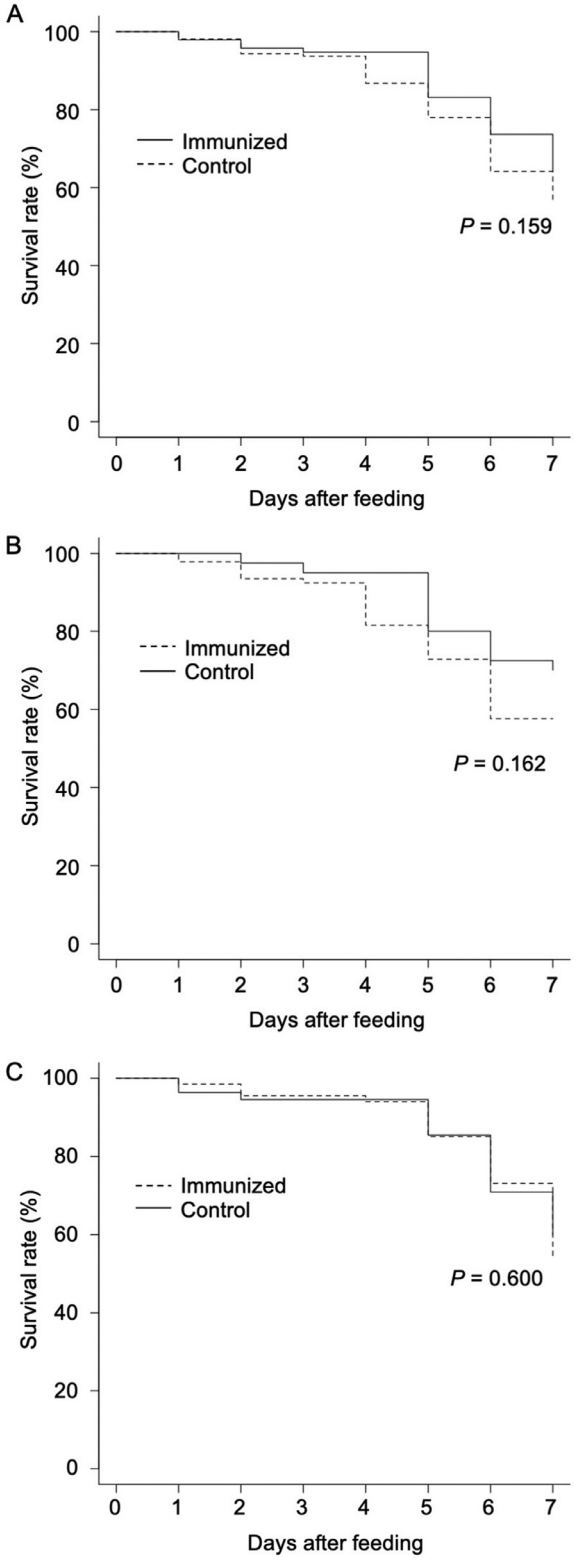

Figure 7. Survival of adult and nymph poultry red mites fed with plasma obtained from chickens immunized with Deg-CPR-2 (experiment 2). Feeding the blood containing the plasma was artificially performed by in vitro feeding assays, and the number of dead PRMs was recorded and plotted on the graph to generate Kaplan−Meier curves. The total number of blood-fed nymphs and adults monitored was as follows: (A) all stages: fed with immune plasma: $n = 159$, fed with control plasma: $n = 95$; (B) adults: fed with immune plasma: $n = 67$, fed with control plasma: $n = 55$); (C) nymphs: fed with immune plasma: $n = 92$, fed with control plasma: $n = 40$. Statistical analysis was performed using a log-rank test. *Results with $P < 0.05$ were considered statistically significant.

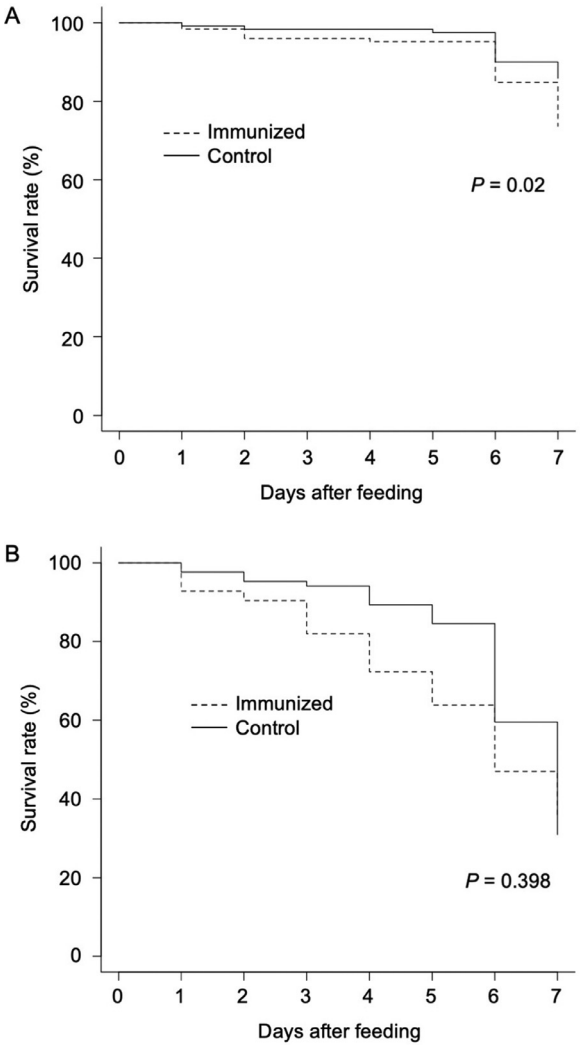

Figure 8. Survival of adult and protonymph poultry red mites fed with plasma obtained from chickens immunized with Deg-CPR-2 (experiment 3). The PRMs were artificially fed with plasma using in vitro feeding assays, and the number of dead PRMs was recorded and plotted on the graph to generate Kaplan−Meier curves. The total number of nymphs and adults fed with plasma was as follows: (A) adults: fed with immune plasma: $n = 125$, fed with control plasma: $n = 120$; (B) protonymphs: fed with immune plasma: $n = 83$, fed with control plasma: $n = 84$. Statistical analysis was performed using a log-rank test. *Results with P values < 0.05 were considered statistically significant.

addition to the *Deg-CPR-2* expression profiles, gene silencing by RNA interference could be a useful tool for investigating the importance of cysteine proteases in each life stage (Chen et al., 2021).

This study characterized Deg-CPR-2, which differs from 2 other previously reported cysteine proteases; moreover, we assessed its potential as a vaccine antigen in vitro. Deg-CPR-2 was predicted to be expressed in the midgut and all life stages of PRMs. The recombinant Deg-CPR-2 showed enzyme activities, which were inhibited in the presence of a CPR inhibitor, suggesting that Deg-CPR-2 functions as a cysteine protease in PRMs. In addition, plasma from chickens immunized with Deg-CPR-2 tended to show acaricidal effects in protonymphs. To date, several candidates for vaccine antigens against PRMs have been identified (Bartley et al.,

Table 5. Cumulative mortality of protonymph poultry red mites fed with plasma obtained from chickens immunized with Deg-CPR-2 (experiment 3).

	Days after feeding						
	1	2	3	4	5	6	7
Protonymphs fed immune plasma ($n = 83$)							
No. of dead protonymphs	6	8	15	23	30	44	54
Mortality (%)	7.23	9.64	18.07	27.71	36.14	53.01	65.06
Protonymphs fed control plasma ($n = 84$)							
No. of dead protonymphs	2	4	5	9	13	34	58
Mortality (%)	2.38	4.765	5.95	10.71	15.48	40.48	69.05
P value (Fisher's exact test)	0.168	0.248	0.018*	0.006*	0.003*	0.122	0.624
Odds ratio	3.17	2.12	3.46	3.17	3.07	1.65	0.84
95% CI	0.55−33.08	0.54−10.05	1.12−12.81	1.30−8.40	1.40−7.07	0.86−3.21	0.42−1.67

Abbreviation: CI, confidence interval.
*P values < 0.05 were considered statistically significant.

2009,2012,2015; Wright et al., 2009,2016; Lima-Barbero et al., 2019a,b; Tatham et al., 2019; Xu et al., 2020; Murata et al., 2021). These antigens were assessed by in vitro feeding assays or on hen feeding assays (Nunn et al., 2020). In addition, the conditions of the in vitro feeding assays were different in each study, making it difficult to compare the effects of the different candidate vaccine antigens identified. However, as Deg-CPR-2 could potentially suppress protonymph growth, it could be included in a cocktail vaccine to enhance the effects of the other antigens. To develop better vaccines, the effects of cocktail vaccines containing various antigen combinations should be assessed. To evaluate the vaccine efficacy in chickens, the mortality and fecundity should be assessed using PRM-challenge trials on immunized chickens.

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DISCLOSURES

TS, EO, and AT are employed by Vaxxinova Japan K.K, Tokyo, Japan. TA, SM, SF, TS, EO, NM, TO, SK, and KO are authors of patent-pending materials and techniques described in this manuscript (2021-080691). The other authors have no financial conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2021.101638.

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