


## ORIGINAL ARTICLE

# Identification of an antilymphocyte transformation substance from *Pasteurella multocida*

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## Abstract

*Pasteurella multocida* is one of the most important bacteria responsible for diseases of animals. Crude extracts from sonicated *P. multocida* strain Dainai-1, which is serotype A isolated from bovine pneumonia, were found to inhibit proliferation of mouse spleen cells stimulated with Con A. The crude extract was purified by cation and anion exchange chromatography and hydroxyapatite chromatography. Its molecular weight was 27 kDa by SDS-PAGE and it was named PM27. PM27 was found to inhibit proliferation of mouse spleen cells stimulated with Con A as effectively as did the crude extract; however, its activity was lost after heating to 100°C for 20 min. PM27 did not directly inhibit proliferation of HT-2 cells, which are an IL-2-dependent T cell line, nor did it modify IL-2 production by Con A-stimulated mouse spleen cells. The N-terminal amino acid sequence of PM27 was determined and BLAST analysis revealed its identity to uridine phosphorylase (UPase) from *P. multocida*. UPase gene from *P. multocida* Dainai-1 was cloned into expression vector pQE-60 in *Escherichia coli* XL-1 Blue. Recombinant UPase (rUPase) tagged with His at the C-terminal amino acid was purified with Ni affinity chromatography. rUPase was found to inhibit proliferation of mouse spleen cells stimulated with Con A; however, as was true for PM27, its activity was lost after heating to 100°C for 20 min. Thus, PM27/UPase purified from *P. multocida* has significant antiproliferative activity against Con A-stimulated mouse spleen cells and may be a virulence factor.

## KEYWORDS

bovine respiratory disease, *Pasteurella*, pathogenicity factor, uridine phosphorylase

**Abbreviations:** BRD, bovine respiratory disease; OMP, outer membrane protein; rUPase, recombinant UPase; UPase, uridine phosphorylase.

## 1 | INTRODUCTION

*Pasteurella multocida* is able to cause diseases in a variety of animal species, examples being hemorrhagic septicemia in cattle, atrophic rhinitis in pigs, fowl cholera in poultry, and pneumonia in many animals. These infections cause large

economic losses to the livestock industry. There are clear correlations between capsular types and specific diseases. For example, Serotype A is associated with fowl cholera, Serotypes B and E with hemorrhagic septicemia, and Serotypes A and D with bovine respiratory disease (BRD).<sup>1,2</sup>

BRD is a generic term that encompasses both pneumonia and shipping fever.<sup>1</sup> *P. multocida* can be both a primary or secondary factor in causation of BRD,<sup>3,4</sup> which is considered to be caused by multiple infectious and environmental factors, stress also playing a major role. The infectious factors for BRD include both viruses and/or bacteria such as bovine herpesvirus-1<sup>5,6</sup>, bovine respiratory syncytial virus,<sup>7,8</sup> parainfluenzavirus-3, bovine coronavirus, bovine viral diarrhoea virus,<sup>9</sup> bovine reovirus, *Mannheimia haemolytica*,<sup>10</sup> and *P. multocida*.<sup>3,9</sup> In the wake of these infections, calves can be infected by other infectious agents such as *P. multocida*, *Histophilus somni*,<sup>11</sup> *Trueperella pyogenes*, and mycoplasma,<sup>12</sup> the resulting pneumonia being more severe. Each pathogen has virulence factors such as adherence factors, toxins, and bacterial components including lipopolysaccharide and the capsule. Therefore, it is considered that both the causes and course of this disease are complicated.

*P. multocida* A:3 is the most common serotype isolated from animals with BRD.<sup>3,4</sup> For infection and colonization, *P. multocida* must first attach to host cell surfaces by adhesion. At this stage, several virulence factors such as fimbriae and outer membrane proteins (OMPs) are involved.<sup>13</sup> *P. multocida* also fight against host immune systems to enable survival in the host. LPS and the capsule are the major factors during this stage. LPS is one of the stimulators of elements of the immune systems, inducing inflammatory cytokines, including IL-1 $\alpha$ , IL-6, and TNF- $\alpha$ .<sup>14</sup> As a result, LPS induce endotoxin shock. The capsule evades phagocytosis and complement-mediated lysis.<sup>15</sup> Adherence factors such as pili play a role in adherence to cell surfaces.<sup>1</sup> OMPs also participate in invasion and intracellular survival to evade of host defense.<sup>16,17</sup> Even though these virulence factors are known, it is still unclear how *P. multocida* exacerbates pneumonia and whether it helps to create multiple infections.

In the present study, we investigated a crude extract obtained from a filtrate of sonicated *P. multocida* that had been isolated from a bovine with respiratory disease. We found that the extract inhibited proliferation of Con A-stimulated mouse spleen cells and therefore sought to identify the responsible protein.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethics statement

Animal experimentation protocols were approved by the President of Kitasato University through a judgment by

the Institutional Animal Care and Use Committee of Kitasato University (Approval no 14-0320).

### 2.2 | Bacterial strain

*P. multocida* Dainai-1 isolated from a bovine with respiratory disease was used in this study. This strain was lyophilized and stored at 4°C. When the strain was used in experiments, the lyophilized organisms were suspended in PBS, inoculated onto 5% sheep blood agar, and cultured at 37°C for 18 hr prior to use.

### 2.3 | Isolation and purification of an antilymphocyte transformation substance

*P. multocida* was inoculated onto 5% sheep blood agar and cultured at 37°C for 18 hr, after which the bacteria were collected and resuspended in 8 mL of PBS. Two mL of the bacterial suspension was inoculated into 200 mL of BHI broth (BD Biosciences, Sparks, MD, USA). Bacteria were cultured at 37°C for 24 hr with shaking, then harvested by centrifugation at 10,000 g for 10 mins at 4°C, washed with 20 mM Tris-HCl (pH 7.5) twice, and lysed by sonication in 20 mM Tris-HCl (pH 7.5). The lysate was cleared by centrifugation at 15,000 g for 10 min at 4°C and then used as a crude extract. The crude extract was loaded onto a Macro-Prep High Q Support (Bio-Rad Laboratories, Hercules, CA, USA) anion exchange column (column diameter 1.0 cm, 2 mL bed volumes) at a flow rate of 1.0 mL/min. After washing with 20 mM Tris-HCl (pH 7.5), proteins were eluted using a 0–500 mM NaCl linear gradient. The fractions responsible for most of the inhibitory activity were pooled and dialyzed against 50 mM acetate buffer (pH 5.0). Then it was applied onto a Macro-Prep High S Support (Bio-rad Laboratories) cation exchange column (column diameter 1.0 cm, bed volume 3 mL) at a flow rate of 1.0 mL/min. After washing with 50 mM acetate buffer (pH 5.0), proteins were eluted using a 0–600 mM NaCl linear gradient. The fractions responsible for most of the inhibitory activity were pooled and dialyzed against 5 mM potassium phosphate buffer (pH 7.5). The dialyzed solution was applied to Hydroxyapatite Bio-Gel HTP Gel (Bio-rad Laboratories; column diameter 1.0 cm, bed volume 3 mL) at a flow rate of 0.5 mL/min. Flow-through was collected during washing with 5-mM potassium phosphate buffer (pH 5.0) and dialyzed against PBS. The inhibitory activity of the dialyzed protein was assessed using a proliferation assay. The purity of the protein, which was designated PM27, was over 95% according to silver stain analysis.

During the purification step, protein concentrations were measured using a Bradford protein assay with BSA as the standard.

## 2.4 | Proliferation assay

Proliferation of mouse spleen cells was measured using a Cell Proliferation ELISA, BrdU (Sigma–Aldrich, St Louis, MO, USA), in accordance with the manufacturer's instructions. In brief, mouse spleen cells isolated from BALB/c mice were subjected to RBC lysis buffer to eliminate red blood cells and plated at  $4 \times 10^6$  cells/well in a 96-well plate with Dulbecco's modified Eagle's medium containing 5% FCS. The spleen cells were then cultured in the presence of either Con A ( $1.0 \mu\text{g}/\text{mL}$ ) or Con A with the extracted protein ( $0.1\text{--}10 \mu\text{g}/\text{mL}$ ) from *P. multocida* for 72 hr, after which the absorbance resulting from lymphocyte proliferation in the samples was measured at 450 nm, with 650 nm as a reference wavelength.

HT-2 cell proliferation was measured using Cell Titer 96 Aqueous One Solution (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. In brief, HT-2 cells were plated at  $1 \times 10^4$  cells/well in a 96-well plate with RPMI 1640 containing 5% FCS and 5 U/mL of rHuIL-2 and then cultured in the presence of various concentrations of PM27 for 48 hr, after which Cell Titer 96 Aqueous One Solution was added to each well. After a 4-hr incubation, the absorbance was measured at 495 nm.

Both results are expressed as ODs and were calculated by subtracting the ODs of unstimulated cultures. Each experiment was assayed in triplicate and all experiments were performed at least three times.

## 2.5 | IL-2 production assay

Mouse spleen cells were prepared as described above. Cells were plated at  $1 \times 10^6$  cells/well in a 24-well plate with Dulbecco's modified Eagle's medium containing 5% FCS and  $1 \mu\text{g}/\text{mL}$  of Con A. Mouse spleen cells were cultured in the presence of  $10 \mu\text{g}/\text{mL}$  of PM27 for 72 hr. The supernatants were collected at 72 hr and stored at  $-30^\circ\text{C}$ , after which IL-2 concentrations were measured.

HT-2 cells were maintained in growth medium with RPMI 1640 containing 5% FCS with rHuIL-2 ( $100 \text{ U}/\text{mL}$ ). HT-2 cells were cultured in growth medium without rHuIL-2 for 24 hr before experiments. After starvation, HT-2 cells were resuspended in growth media without rHuIL-2 and plated at  $1 \times 10^4$  cells/well in a 96-well plate. Collected supernatants prepared as described above were added to each well. The cells were cultured for 48 hr and their proliferation measured using Cell Titer 96 Aqueous

One Solution in accordance with the manufacturer's instructions. Results are expressed as ODs. Each experiment was assayed in duplicate and all experiments were performed at least three times.

## 2.6 | Determination of N-terminal amino acid sequence

The partial N-terminal amino acid sequence of PM27 was determined by automated Edman degradation by Hokkaido System Science (Sapporo, Japan). Similar sequences and alignment were searched for BLAST. The results of N-terminal amino acid sequencing suggested that the PM27 gene of *P. multocida* Dainai-1 is in fact the organism's uridine phosphorylase (*UPase*) gene.

## 2.7 | Cloning and expression

The *UPase* gene was cloned into expression vector pQE-60 (Qiagen GmbH, Hilden, Germany) and transfected into *Escherichia coli* XL-1 Blue. Recombinant *UPase* (r*UPase*) that had been His-tagged at the C-terminal amino acid was purified with Ni affinity chromatography and dialyzed against PBS<sup>-</sup>. The purity of r*UPase* was checked by silver stain analysis on SDS-PAGE. Its activity was measured as described above.

## 2.8 | Statistical analysis

Student's *t*-test was used for comparing results from the proliferation assay. Differences between groups were considered to be statistically significant when  $P < 0.05$ .

# 3 | RESULTS

## 3.1 | Antiproliferative activity of sonicated antigen from *P. multocida*

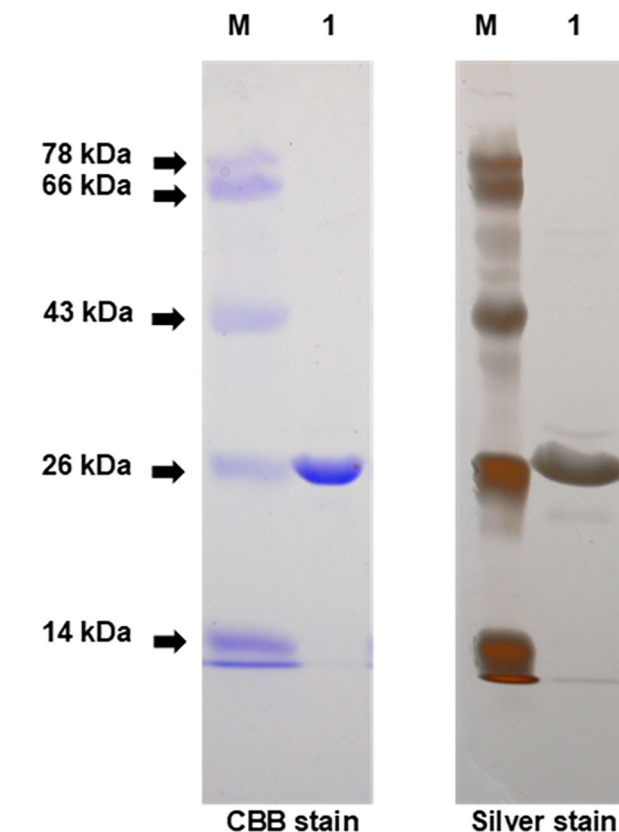
The antiproliferative activity of the crude extract of *P. multocida* was determined by measuring proliferation of Con A-stimulated mouse spleen cells. Compared with proliferation of Con A-stimulated mouse spleen cells, 10, 1, and  $0.1 \mu\text{g}/\text{mL}$  of the crude extract reduced proliferative activity by  $43.2\% \pm 4.9$ ,  $13.8\% \pm 4.5$ , and  $0.3\% \pm 6.2$ , respectively. In contrast, heat-treated crude extract that was heated to  $100^\circ\text{C}$  for 20 min did not inhibit proliferative activity (data not shown). It was therefore presumed that a heat-labile protein in the crude extract was responsible for suppressing proliferation of Con A-stimulated mouse spleen cells.

### 3.2 | Purification of antilymphocyte transformation substance

The crude extract was fractionated using anion exchange chromatography (Figure 1a). Proteins were eluted using a 0–500 mM NaCl linear gradient. Because the peak of the protein fractions was a broad single peak, inhibitory activity was measured for every third fraction. Inhibitory activity was observed from fraction numbers 21 to 30, eluted at 135.1–195.9 mM NaCl. The inhibitory activity of these fractions was 30.0–56.3%; the other fractions showed no significant inhibitory activity. Thus, these fractions were collected and subjected to cation exchange chromatography (Figure 1b). Proteins were eluted at 0–600 mM NaCl. The peak of the protein fractions was a broad peak. Fraction numbers 48–57, eluted at 477.9–569.5 mM NaCl, exhibited inhibitory activity of 48.7–84.7%. According to SDS-PAGE, there was one major band at 27 kDa and a few minor bands in these fractions (data not shown). These were further fractionated by hydroxyapatite chromatography. The 27-kDa protein did not bind to the column and was therefore was collected in the flow-through. The molecular weight of the collected protein was approximately 27 kDa according to Coomassie brilliant blue staining (Figure 2). The purity of this protein was higher than 95% according to Coomassie brilliant blue and silver staining. This 27-kDa protein was designated as PM27.

### 3.3 | Antiproliferative activity by PM27

The antiproliferative activity of PM27 was measured against Con A-stimulated mouse spleen cells (Figure 3). PM27 reduced cell proliferation by  $57.6\% \pm 9.0$  and  $19.9\% \pm 5.0$  at concentrations of 10 and  $1\ \mu\text{g}/\text{mL}$  compared with Con A-stimulated mouse spleen cells. In comparison, PM27 heated at  $100^\circ\text{C}$  for 20 min lost its inhibitory activity. PM27 showed the same activity as did

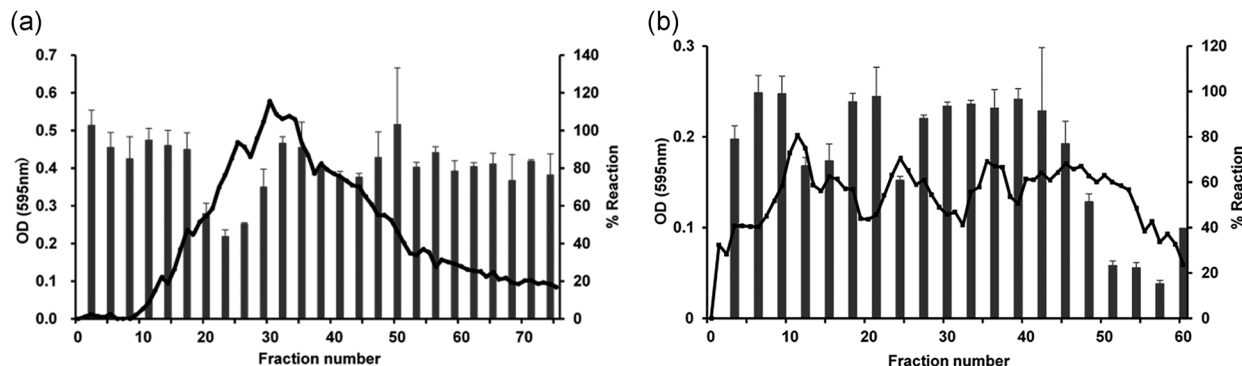


**FIGURE 2** SDS-PAGE of purified 27 kDa protein. Lane M: Indicated molecular mass markers. Lane 1: Purified protein from crude extract of *P. multocida*

the crude extracts. These observations suggest that PM27 is the target protein in the crude extract and is heat-labile.

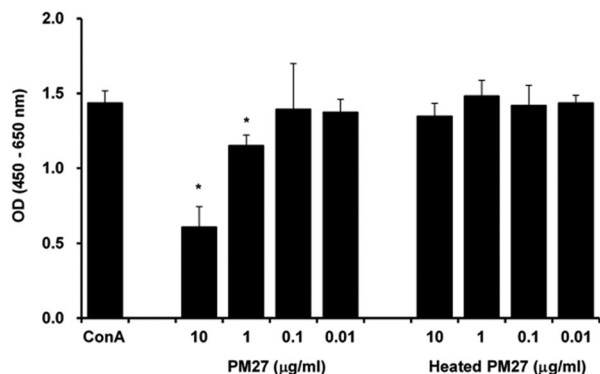
### 3.4 | Inhibition of HT-2 cell proliferation by PM27

Con A is known to be a T cell mitogen in mice. Therefore, whether PM27 inhibits T cells directly or indirectly was



**FIGURE 1** Separation of sonicated antigen from *P. multocida* by chromatography. The bar graph shows the reaction rate, which is the activity value when the control value is taken as 100%. The line graph shows the amount of protein in each fraction by its absorbance value (595 nm). (a) Anion exchange chromatography of the preparation of sonicated *P. multocida*. (b) Cation exchange chromatography of the preparation from anion exchange chromatography





**FIGURE 3** Proliferation in ConA-stimulated mouse spleen cells cultured with PM27 or heated PM27. Mouse spleen cells that had been stimulated with Con A (1 μg/mL) were cultured with p-PM27 or heated p-PM27. Data are expressed as mean OD ± SD. \* $P < .05$

investigated using murine HT-2 cells, an IL-2-dependent T cell line. HT-2 cells were cultured with 5 U/mL rHuIL-2 with or without various concentrations of PM27 (Figure 4). PM27 did not inhibit HT-2 cell growth, even when it was added at concentrations that are high enough to inhibit Con A-stimulated mouse spleen cells. These results suggest that PM27 does not inhibit murine T cells directly.

### 3.5 | Effect of IL-2 production by PM27

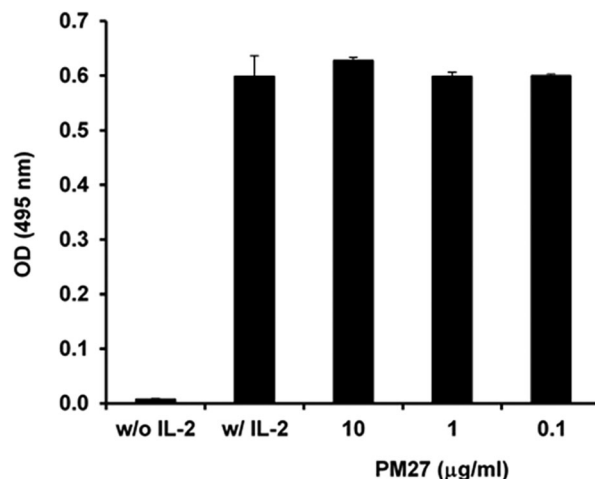
IL-2 stimulates T cells and induces their proliferation. Hence, PM27 may inhibit IL-2 production. To investigate this possibility, the culture supernatant was collected from Con A-stimulated mouse spleen cells with or without PM27 and IL-2 production by HT-2 cell proliferation measured (Figure 5). There was no difference in the results with or without PM27, suggesting that PM27 does not inhibit IL-2 production.

### 3.6 | N-terminal amino acid sequencing

The sequence of residues of PM27 was determined to be SEVFHLGLTK by Pehr Edman's automated degradation method. BLAST search found this sequence to be identical to that of UPase from *P. multocida*.

### 3.7 | Cloning and expression of UPase as PM27

The UPase gene was cloned from *P. multocida* Dainai-1 into expression vector pQE-60 in *Escherichia coli* XL-1 Blue. rUPase was purified with Ni affinity chromatography and then compared with purified PM27 from *P. multocida* Dainai-1 by SDS-PAGE (Figure 6a); Its

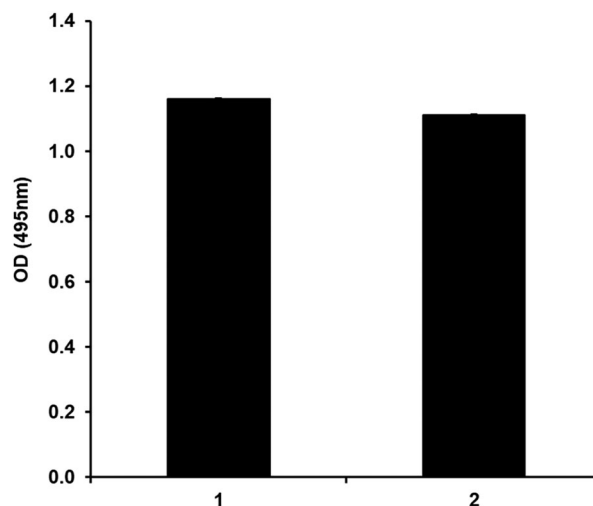


**FIGURE 4** Effect of PM27 on proliferation of HT-2 cells. HT-2 cells were cultured with purified PM27. Proliferative activity was measured after 48-hr culture

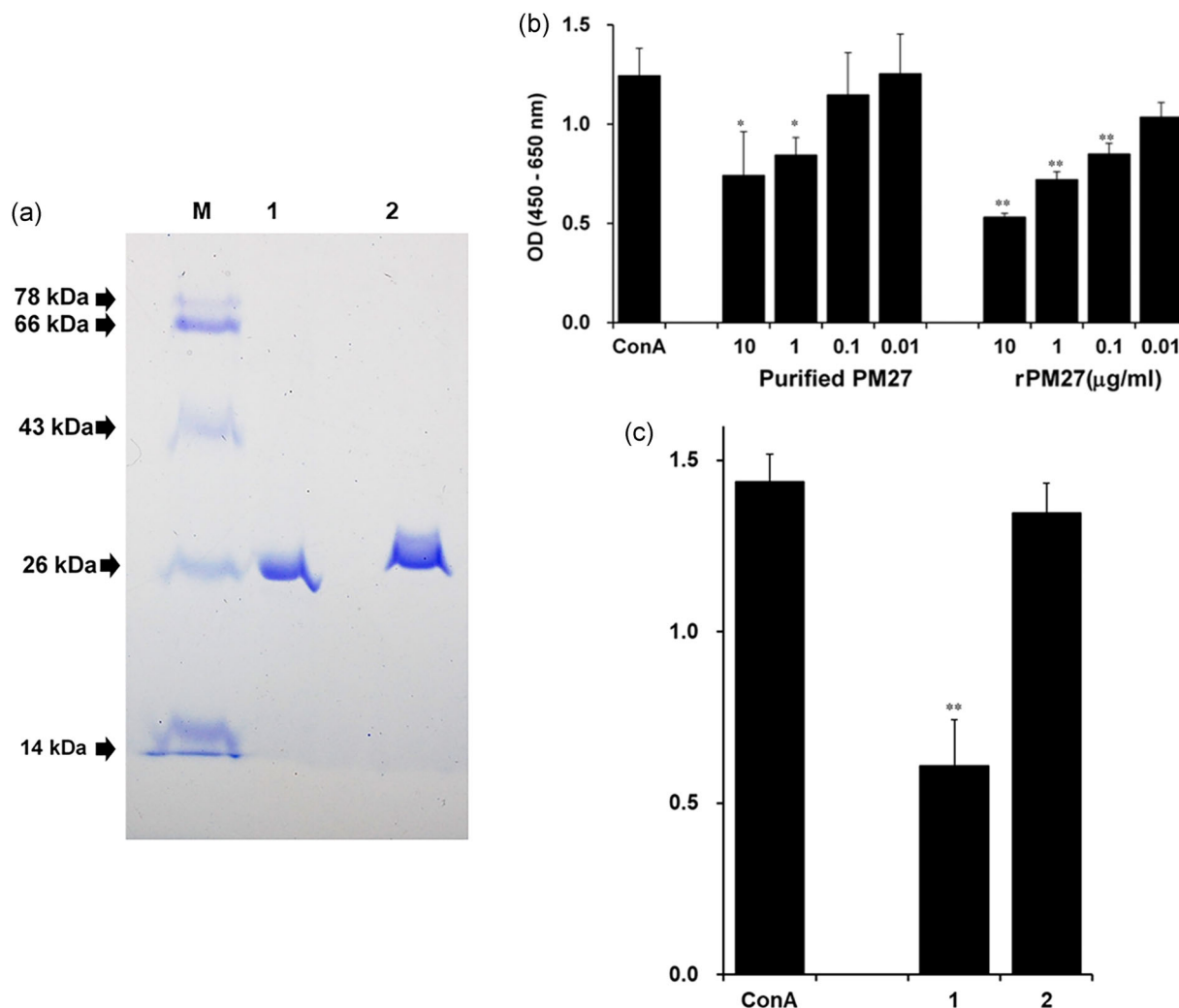
molecular weight was found to be slightly greater, because rUPase has an extra amino acid, His, at the C-terminal amino acid. Its antiproliferative activity against Con A-stimulated mouse spleen cells (Figure 6b) was then measured. rPM27 reduced cell proliferation at a slightly higher rate than did purified PM27 from *P. multocida* Dainai-1. rUPase also lost activity after heat treatment (Figure 6c).

## 4 | DISCUSSION

*P. multocida* causes various diseases in various animals. Various virulence factors of *P. multocida* have already been reported. In this study, a crude extract from



**FIGURE 5** Effect of rPM27 on IL-2 production. HT-2 cells were cultured with supernatant from spleen cells cultured with Con A (lane 1) or Con A and rPM27 and their proliferative activity measured



**FIGURE 6** Comparison of purified PM27 and rPM27. (a) SDS-PAGE of p-PM27 and rPM27. SDS-PAGE was performed with 1  $\mu$ g of purified PM27 (Lane 1) and rPM27 (Lane 2). (b) Proliferation in ConA-stimulated mouse spleen cells with purified PM27 or rPM27. (c) Proliferation in ConA-stimulated mouse spleen cells with 10  $\mu$ g/mL of rPM27(#1) or heat-treated rPM27(#2). \* $P < .05$ , \*\* $P < .01$

*P. multocida* showed antiproliferative activity against Con A-stimulated mouse spleen cells. Known virulence factors of *P. multocida* include *P. multocida* toxin,<sup>18</sup> OMPs, capsule, pili, and LPS.<sup>1</sup> LPS and OMPs have been shown to modulate immune system responses by inducing proinflammatory cytokines.<sup>14</sup> However, none of them has been reported to show antiproliferative activity. Although the crude extract may have been contaminated with LPS, the heat-treated crude extract lost activity, indicating that the causative agent was likely a heat-labile protein and not LPS.

An approximately 27-kDa protein, PM27, was purified by using anion and cation exchange chromatography and hydroxyapatite chromatography. PM27 showed the same antiproliferative activity as did the crude extract. Its activity was lost after heating to 100°C for 20 min. These findings point to PM27 as the causative agent. In a

comparison using molecular weight as an indicator, the molecular weight of *P. multocida* toxin was 146 kDa,<sup>19,20</sup> whereas the molecular weights of OmpA and OmpH were 35.4–38.5 kDa and 37.5 kDa, respectively.<sup>21</sup> Therefore, the *P. multocida* toxin was considered to differ from virulence factors that have been reported previously. However, it was also possible that PM27 is a larger, known virulence factor that was broken down after production or during the purification process. N-terminal amino acid analysis of PM27 was therefore performed to determine the identity of PM27 and rule out that possibility. BLAST search results indicated that PM27 is the *UPase* gene from *P. multocida*. UPases have been identified from prokaryotes to eukaryotes and are known to be key enzymes in the pyrimidine salvage pathway, which catalyzes the reversible phosphorolysis of uridine to uracil and ribose 1-phosphate.<sup>22,23</sup> However, UPase

has not been reported to show antiproliferative activity. To confirm that, a rUPase was constructed and its activity analyzed; the findings indicated that UPase has the same antiproliferative activity as the original purified protein. It has been reported that de novo pyrimidine biosynthesis is required for the virulence of *Toxoplasma gondii*.<sup>24</sup> Therefore, it has been investigated the possibility of attenuated vaccine using de novo pyrimidine biosynthesis pathway deficiency strain.<sup>25,26</sup> However, that UPase from bacteria plays a role as a virulence factor has not previously been reported. Thus, our study identified a new activity for UPase from *P. multocida*.

According to the results of cell proliferation using HT-2 cells and IL-2 production assays, PM27 does not inhibit HT-2 cells directly and does not affect IL-2 production by Con A-stimulated mouse spleen cells. Thus, PM27 may not act directly on T cells but rather inhibit them by inducing production of other cytokines such as IL-10 or TNF- $\alpha$  by other cells.

In this study, we found that a 27-kDa heat-labile protein purified from sonicated *P. multocida* inhibits proliferation of Con A-stimulated mouse spleen cells. Amino acid analysis revealed PM27 to be the UPase gene from *P. multocida*. Whether the activity of UPase is involved in pathogenicity was not clarified from this study. However, all bacteria, gram-positive and negative alike, possess this enzyme; hence, whether many bacteria have antiproliferative activity and whether that activity differs between strains of *P. multocida* requires further investigation.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to declare.

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