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Possible molecular mechanism for acute encephalopathy by angel-wing mushroom ingestion – Involvement of three constituents in onset –

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ABSTRACT

In Japan in 2004, 59 people who had consumed angel-wing mushroom, *Pleurocybella porrigens*, experienced acute encephalopathy, and of these 17 died. We purified a lethal protein to mice, pleurocybelline (PC), from *P. porrigens*. Although PC caused no damage to the brain, PC formed a complex with a lectin (PPL) and showed exo-protease activity, degrading substrates from both *N*- and *C*-termini. In addition, the presence of an unstable toxic compound, pleurocybellaziridine (PA), in the mushroom was demonstrated. We hypothesized that the complex and PA are involved in disease development and verified that apoptotic cells in the hippocampus were significantly increased by injection of the mixture of PC, PPL, and PA, indicating that these substances might be involved in acute encephalopathy.

1. Introduction

The mushroom *Pleurocybella porrigens* (angel's wing mushroom in English; Sugihiratake in Japanese) belongs to the family Tricholomataceae and is the only species of fungus in the genus *Pleurocybella* (Matsumoto et al., 2005). This common mushroom is widespread throughout the world's temperate regions and grows on dead trees such as cedar and pine (Ainsworth et al., 2001). The fruiting body has long been consumed throughout the world and was considered a delicacy due to its distinct flavor and aroma. However, in the autumn of 2004 in Japan, 59 incidents of food poisoning were reported following consumption of *P. porrigens*, and 17 people died from acute encephalopathy (Kuwabara et al., 2005; Obara et al., 2005, 2008). Moreover, prior to death, the patients had undergone hemodialysis due to chronic renal failure. As a result, the Ministry of Health, Labour and Welfare of Japan convened a study group; however, the team concluded that the disease was of unexplained origin and the group was dissolved in 2006. The corresponding author (HK) of this study was a member of the study group and we have been continuing to research the cause of the food poisoning since then.

To date, some research groups have reported constituents of *P. porrigens* such as vitamin D analogs (Sasaki et al., 2006), fatty acids (Hasegawa et al., 2007), saccharides (Takata et al., 2009), and hydrogen cyanide (Gonmori and Yokoyama, 2009) as potential causative toxins for the food poisoning. Previously, we succeeded in the purification, characterization, and heterologous expression of a lectin, *P. porrigens* lectin (PPL) (Suzuki et al., 2009, 2014, 2021). Further, some novel toxic amino acid derivatives were isolated by us (Kawaguchi et al., 2009). We predicted the presence of an unstable precursor of the amino acid derivatives based on their structures, demonstrated the existence of the precursor in *P. porrigens*, and named it pleurocybellaziridine (PA). PA has an unstable aziridine ring and was observed to suppress cellular

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viability of cultured oligodendrocytes (Wakimoto et al., 2010).

Moreover, whole-genome and transcriptome sequencing of *P. porrigens* has been undertaken using next-generation sequencing technology, and the integrated genome database, A-WING, was constructed (Suzuki et al., 2013; Yamamoto et al., 2014). The results of genome and transcriptome analyses revealed that the fungus has a unique genome signature and contains numerous novel genes compared with other basidiomycetes (Suzuki et al., 2013). Even though studies on *P. porrigens* have been conducted using various approaches as mentioned above, the mechanism of acute encephalopathy following *P. porrigens* consumption remains to be elucidated.

The goal of this study was to elucidate the molecular mechanism of acute encephalopathy caused by *P. porrigens* intake. In the present study, we attempted to isolate a substance lethal to mice, and successfully purified a novel glycoprotein, pleurocybelline (PC). Furthermore, we hypothesized that the complex of two proteins, PPL and PC, shows protease activity and damages the blood brain barrier (BBB), after which PA attacks the brain and causes acute encephalopathy. To validate this hypothesis, we performed histological analysis by immunostaining after intraperitoneal administration of various combinations of the three substances.

2. Materials and methods

2.1. Materials

Fruiting bodies of *P. porrigens* were collected from Yamanashi Prefecture, Japan, and stored frozen at −20 °C until use. For use as substrates in the protease assay, insulin (human, recombinant), bovine serum albumin (BSA), and Immunoglobulin G (IgG) were purchased from Fujifilm Wako Pure Chemical Co., Ltd. (Tokyo, Japan) and Merck Co. (Darmstadt, Germany), while PPL (Suzuki et al., 2009), and pleurocybellaziridine (PA) (Wakimoto et al., 2010) used in this study were manually purified and synthesized, respectively. Recombinant PPL (rPPL) used in this study was expressed in *Escherichia coli* using the pCold I DNA vector as described previously (Suzuki et al., 2021).

2.2. Toxicity test

All experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee of Utsunomiya University of Japan (Approval No. A17-0010) and performed in accordance with the approved guidelines and regulations. The authors complied with the ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines). Five-week-old male ddY mice were purchased from Japan SLC Co. Ltd. (Shizuoka, Japan). The samples were dissolved in 10 mM sodium phosphate-buffered solution, pH 7.4, (PBS) and then intraperitoneally administered to the mice once. The injected volume was 10 ml/kg body weight (BW). In order to administer the samples in various combinations, the doses of each sample were set as follows: PC, 24 mg/kg BW; PPL, 30 mg/kg BW; PA, 70 mg/kg BW. The dose of PC (24 mg/kg) was set at a concentration that was lethal to mice. Although single administration of PPL (30 mg/kg) did not show lethal activity to mice, its concentration was set to be equal to PC in molar ratio. The dose of PA (70 mg/kg), which was also not lethal to mice, was set to be in excess of the molar concentrations of PC and PPL.

2.3. Purification of PC from P. porrigens

Purification of PC was guided by its toxicity to mice as an indicator. Lyophilized fruiting bodies of *P. porrigens* were extracted with distilled water (25 °C, overnight) and then boiling water (100 °C, 30 min). The boiling water-soluble part was fractionated into high- and low-molecular-weight fractions by dialysis and both fractions were lyophilized.

Three grams of the high-molecular-weight fraction was dissolved in 10 mM phosphate buffer (pH 7.5) and subjected to anion-exchange chromatography (DEAE-Toyopearl, Tosoh, Tokyo, Japan) equilibrated with the same buffer. The adsorbed materials were eluted stepwise with a buffer containing 0.1, 0.2, 0.3, 0.4, 0.5, and 1 M NaCl. The eluent (0–0.2 M NaCl), which showed lethal activity in mice, was dissolved in 10 mM acetate buffer (pH 4.5) and subjected to cation exchange chromatography (Toyopearl CM-650). The column was washed with the same buffer and then eluted in a stepwise manner with different concentrations of NaCl (0.1, 0.2, 0.3, 0.4, 0.5, and 1 M) in the buffer. Ammonium sulfate was added to the non-adsorbed fraction at a final concentration of 30%, the sample was stirred overnight, and then centrifuged (8500×*g* for 20 min). The obtained precipitates were dissolved in PBS and further applied to gel filtration chromatography (Sephacryl S200HR, Cytiva, Tokyo, Japan) equilibrated with the same buffer. Finally, the fraction that showed lethal activity in mice was dissolved in 10 mM phosphate buffer (pH 7.4) containing 4.3 M ammonium sulfate and subjected to hydrophobic interaction chromatography (Toyopearl Butyl-650 M, Tosoh). The column was washed with the same buffer, and the bound material was eluted stepwise with 2.9, 1.8, and 0.8 M ammonium sulfate in the starting buffer and fractionated into fractions 1 to 11. The fraction that showed lethality to mice was designated as PC.

2.4. Protease assay

Insulin, BSA, and IgG were used as substrates for the protease assay. PC (0.75 mg/ml), PPL (0.75 mg/ml), and each substrate (0.25 mg/ml) were dissolved in PBS and incubated at 37 °C for 72 h. This reaction mixture was desalted and concentrated (10-fold) using Amicon Ultra-0.5 centrifugal ultrafiltration units (Merck). The reaction products were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis (AutoFlex II, Bruker, Billerica, MA, USA). The protease activity of rPPL expressed in *E. coli* (Suzuki et al., 2021) was confirmed using the same method as described above (Fig. S11).

2.5. Molecular mass determination

The molecular mass of PC was determined using SDS-PAGE and MALDI-TOF MS spectrometry. SDS-PAGE was performed using a 15% polyacrylamide slab gel in the presence of SDS (Laemmli, 1970). XL-Ladder Low (APRO Science, Tokushima, Japan) marker was used as the molecular weight marker in the Coomassie Brilliant Blue (CBB) staining. In order to detect sugars and proteins, fluorescence staining was performed with a Pro-Q Emerald 300 Glycoprotein Gel Stain Kit (Molecular Probes, Eugene, OR, USA) and a SYPRO Ruby Protein Gel Stain Kit (Molecular Probes) according to the manufacturer's instructions. CandyCane™ glycoprotein molecular weight standards (Molecular Probes) were used as markers in fluorescence staining. MALDI-TOF MS analysis was conducted using an AutoFlex II spectrometer (Bruker), as previously described (Suzuki et al., 2009).

2.6. Immunohistochemistry

In order to examine the effect of the three compounds on the brain, various combinations were intraperitoneally administered to mice with three biological replicates. The treatment groups were as follows: Control, PBS; PC, PC (24 mg/kg); PPL, PPL (30 mg/kg); PA, PA (70 mg/kg); PC + PPL, PC (24 mg/kg) and PPL (30 mg/kg); PC + PPL + PA, PC (24 mg/kg), PPL (30 mg/kg) and PA (70 mg/kg). Seventy-two hrs after administration, mice were transcardially perfused with 4% paraformaldehyde (PFA) in PBS under anesthesia with isoflurane, and then the brains were collected. Immunostaining was subsequently performed according to the method described previously (Maekawa et al., 2018) with minor modifications using five primary antibodies to neuronal nuclear antigen (NeuN), glial fibrillary acidic protein (GFAP), glucose

transporter 1 (Glut1), microtubule associated protein 2 (MAP2), and single strand DNA (ssDNA).

Brain samples were further fixed in 4% PFA in PBS for 24 h and subsequently incubated in 30% sucrose in PBS for 1 week at 4 °C. Brain tissues were cut with a freezing microtome (REM-710, Yamato, Saitama, Japan) through the coronal plane to generate 50-μm thick slices. Sections were immersed in 0.6% H_2O_2 in PBS for 30 min at room temperature (25 °C), washed twice in PBS, and then blocked with PBS containing 1% casein and 0.1% Triton X-100 for 1 h. Then, each section was incubated overnight at room temperature (20–25 °C) with the five antibodies in PBS containing 1% casein and 0.1% Triton X-100. The five antibodies were: NeuN (1:200 dilution, Merck), GFAP (1:200 dilution, Dako, Glostrup, Denmark), Glut1 (1:1000 dilution, Dako), MAP2 (1:200 dilution, Merck), and ssDNA (1:800 dilution, Dako). The sections were washed four times with PBS, and secondary antibody incubation was performed using the Dako REAL EnVision Detection System (Peroxidase/DAB, rabbit/mouse) for 1 h at room temperature (25 °C). Finally, the sections were washed four times with PBS and stained with 3,3′-diaminobenzidine (DAB) chromogen (Dako). The digital photomicrographs were taken on the microscopes Leica M205 FA (for 10 × magnification, Leica, Wetzlar, Germany) and Olympus B×60 (for 100 × and 200 × magnification, Olympus, Tokyo, Japan). The number of apoptotic cells detected with anti-ssDNA antibody in the whole hippocampus was counted manually, and these calculations were performed by a blinded observer.

2.7. Confirmation of rPPL in the mouse brain

The samples were dissolved in PBS and then intraperitoneally administered to mice once. The volume injected was the same as described above (10 ml/kg BW). The treatment groups were as follows: Control, PBS; rPPL, rPPL (30 mg/kg); PC + rPPL, PC (24 mg/kg) and rPPL (30 mg/kg). The rPPL and PBS treatment groups were used as negative controls. All experiments were performed with three biological replicates. Seventy-two hrs after administration, the mouse brains were collected and immunohistochemical analysis was performed according to the same method described above. An Anti-6 x His-tag antibody (Funakoshi, Tokyo, Japan) was used as the antibody for the analysis.

2.8. Statistical analysis

For comparison between groups, one-way analysis of variance (ANOVA) followed by the Turkey-Kramer multiple comparison test was used. All statistical analyses were performed using R ver. 3.5.2 software. The significance threshold was set at 0.05.

3. Results and discussion

3.1. Purification of lethal substance from P. porrigens

First, we attempted to isolate the substance in *P. porrigens* that is lethal to mice. The lyophilized fruiting bodies of *P. porrigens* were extracted with water at room temperature and then boiling water, and both the extracts showed lethal activity at 3000 mg/kg. These extracts were divided into high- and low-molecular-weight fractions using dialysis, respectively, and both of the high-molecular-weight fractions showed lethal activity (1000 mg/kg). Further, the high-molecular-weight fraction extracted with water at room temperature was boiled for 30 min, and the boiled fraction maintained its toxicity. The results indicated that the lethal toxin(s) was heat-stable and high-molecular-weight. Therefore, the high-molecular-weight fraction extracted with boiling water was further fractionated using anion-exchange chromatography (Toyopearl DEAE-650), followed by cation exchange chromatography (Toyopearl CM-650) and ammonium sulfate precipitation. Since the 30% ammonium sulfate precipitate showed lethal activity, it was further fractionated using gel filtration (Sephacryl S200HR) and hydrophobic interaction chromatography (Butyl Toyopearl), producing a lethal compound (lethal activity, 24 mg/kg) showing a single band on SDS-PAGE. The toxin was named pleurocybelline (PC). Staining of PC in the SDS-PAGE indicated that the toxin was a glycoprotein with a molecular mass of about 10 kDa as a monomer (Fig. 1). N-terminal amino acid sequencing and LC-MS/MS analysis were performed to identify the primary structure of the protein. However, the sequencing failed, since the glycoprotein was only slightly soluble in the solvents and buffers employed. PC showed lethal activity against mice as described above; however, histological analysis of the mouse brains indicated that there were no changes. Furthermore, intraperitoneal administration of PPL (30 mg/kg) or PA (70 mg/kg) did not show lethal activity against mice nor did it cause any damage to brain tissues.

3.2. Protease activity of PC and PPL in combination

Generally, the BBB blocks the transfer of pathogens, other harmful agents, and high-molecular-weight substances from the blood into the brain (Abbott et al., 2010). We hypothesized that PC and PPL in combination shows protease activity and disrupts the BBB. Our hypothesis is based on two observations. The first is the onset mechanism of influenza encephalopathy, in which trypsin-like proteases are produced following viral infection and disrupt the BBB (Kido et al., 2012; Yao et al., 2003). The second is that PPL shows structural similarity to ricin B chain and hemagglutinin component (HA1) of botulinum toxin. While ricin B chain and HA1 are themselves non-toxic, they form complexes with ricin and botulinum toxin as the toxic principles, respectively, and these complexes exhibit toxicity against animals including humans.

Thus, PC and PPL were combined and the protease activity of the mixture was examined using BSA, IgG, and insulin as substrates. The activity was confirmed by SDS-PAGE. The band of BSA (66 kDa) showed no change following treatment with PC or PPL (Fig. 2A, lanes 4 and 6). On the other hand, the band disappeared following treatment with PC and PPL in combination (Fig. 2A, lane 7). The experiment using IgG also produced a similar result (Fig. S1). These results indicated that the mixture of PC and PPL exhibited protease activity. Characterization of

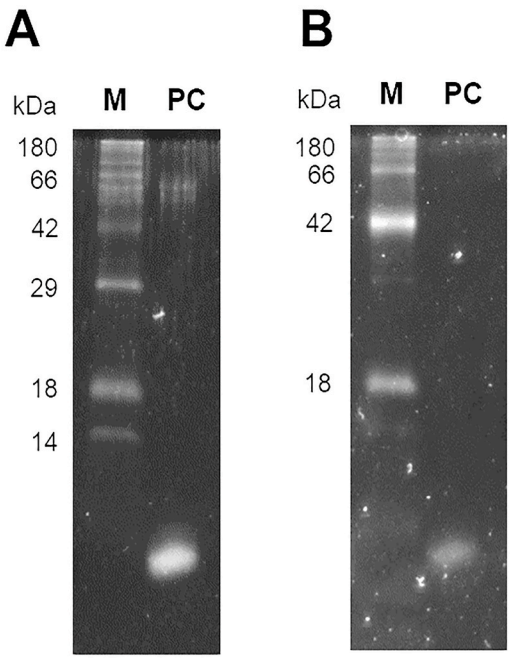

Fig. 1. SDS-PAGE analysis of PC. The gel was stained with a SYPRO Ruby Protein Gel Stain Kit (A) and Pro-Q Emerald 300 Glycoprotein Gel Stain (B). M indicates molecular mass standards (CandyCane™ glycoprotein molecular weight standards).

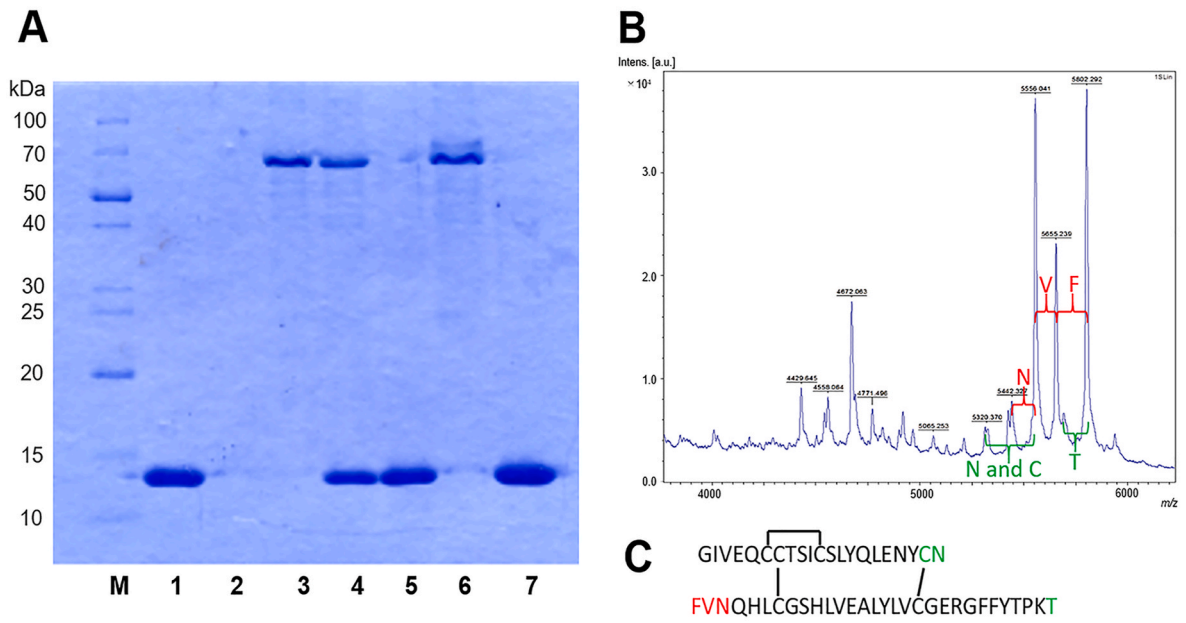

Fig. 2. Protease activity of PC and PPL in combination. (A) SDS-PAGE following protease assay using BSA as a substrate. Lane 1: PPL, lane 2: PC, lane 3: BSA, lane 4: PPL and BSA, lane 5: complex (PC and PPL), lane 6: PC and BSA, lane 7: complex (PC and PPL) and BSA, M: molecular mass standards (XL-Ladder Low). (B) MS spectrum of insulin hydrolyzed by PC and PPL in combination. (C) Primary structure of insulin. Hydrolyzed *N*- and *C*-terminal amino acids deduced from these molecular masses are shown in red and green, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the protease activity of the complex was analyzed with insulin as a substrate using MALDI-TOF MS analysis. The complex started to degrade insulin (*m/z* 5802) following 9 h of incubation, and many degraded molecular ions were observed after 72 h (Fig. S2). Interestingly, the analysis indicated that this complex showed exo-protease activity with no amino acid specificity and degraded the substrate from both *N*- and *C*-termini (Fig. 2B and C and Fig. S3). To the best of our knowledge, a protease completely lacking substrate specificity at both termini has not been previously reported.

3.3. Immunohistochemical analysis

Nomoto et al. reported that demyelinating symptoms might have developed in the brains of patients who consumed *P. porrigens* (Nomoto et al., 2007). Based on the results of previous studies and our findings, we hypothesized that the complex of PC and PPL destroyed or functionally disrupted the BBB, PA then passed through the BBB and showed specific toxicity to the brain cells, resulting in acute encephalopathy (Fig. 3). The vulnerability of hippocampal neurons to a variety of stresses and pathological conditions has been shown in humans and rodents (Bartsch and Wulff, 2015). Hence, the effect of PC, PPL, and PA singly or in various combinations on mouse brain tissues was examined by immunostaining. As a result, distinct immunostaining signals for ssDNA were observed in the hippocampus of mice administered the three compounds in combination, but were not observed in the other samples (Fig. S4). The number of apoptotic cells showing anti-ssDNA antibody immunostaining in the hippocampus was significantly increased in the three-compound mixture group compared with that in the control and the other groups (Fig. 4A). Furthermore, immunohistochemical analysis was performed to detect the transfer of His-tagged rPPL into the mouse brain after administration of PC and rPPL. The results of immunostaining with anti-His tag antibody showed a distinct increase in staining intensity in the two-compound mixture group (i.e., PC and rPPL) compared with that in the PBS (Control) and rPPL groups (Fig. 4B and Fig S5). These results indicate that the combined administration of PC and PPL, which showed protease activity, increased the BBB permeability, resulting in the detection of rPPL in the hippocampus.

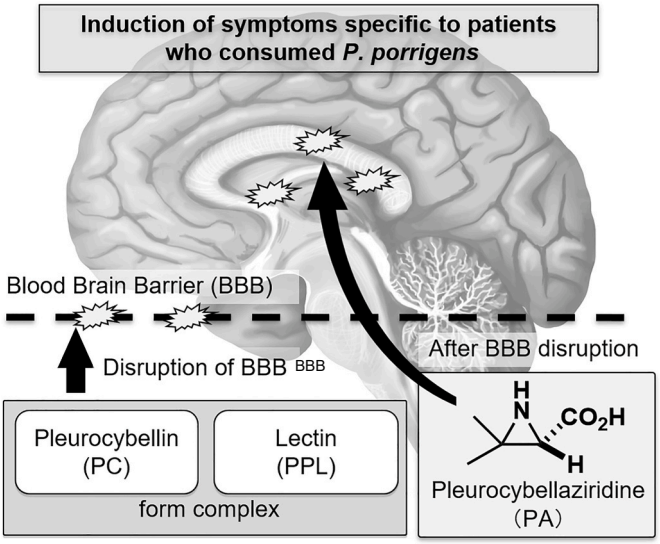

Fig. 3. Proposed molecular mechanism of acute encephalopathy following ingestion of *Pleurocybella porrigens*.

A slight increase in staining intensity of rPPL was observed in the hypothalamus (Fig. 4C and Fig. S6). The results indicate higher staining intensity in both the hypothalamus and hippocampus when the mixture of PC and rPPL was administered (Fig. 4B and C, Fig S5 and Fig S6). In other words, administration of the mixture is likely to cause damage both inside and outside the BBB as well. On the other hand, administration of rPPL alone tended to increase the staining intensity in the hypothalamus, although not significantly so. The relationship between the permeability of the BBB and rPPL treatment alone needs to be further investigated in the future.

In addition, PC, PPL, and PA, singly or in various combinations, did not obviously increase ssDNA-immunopositive numbers in the other brain regions (data not shown). Immunostaining with anti-NeuN, MAP2,

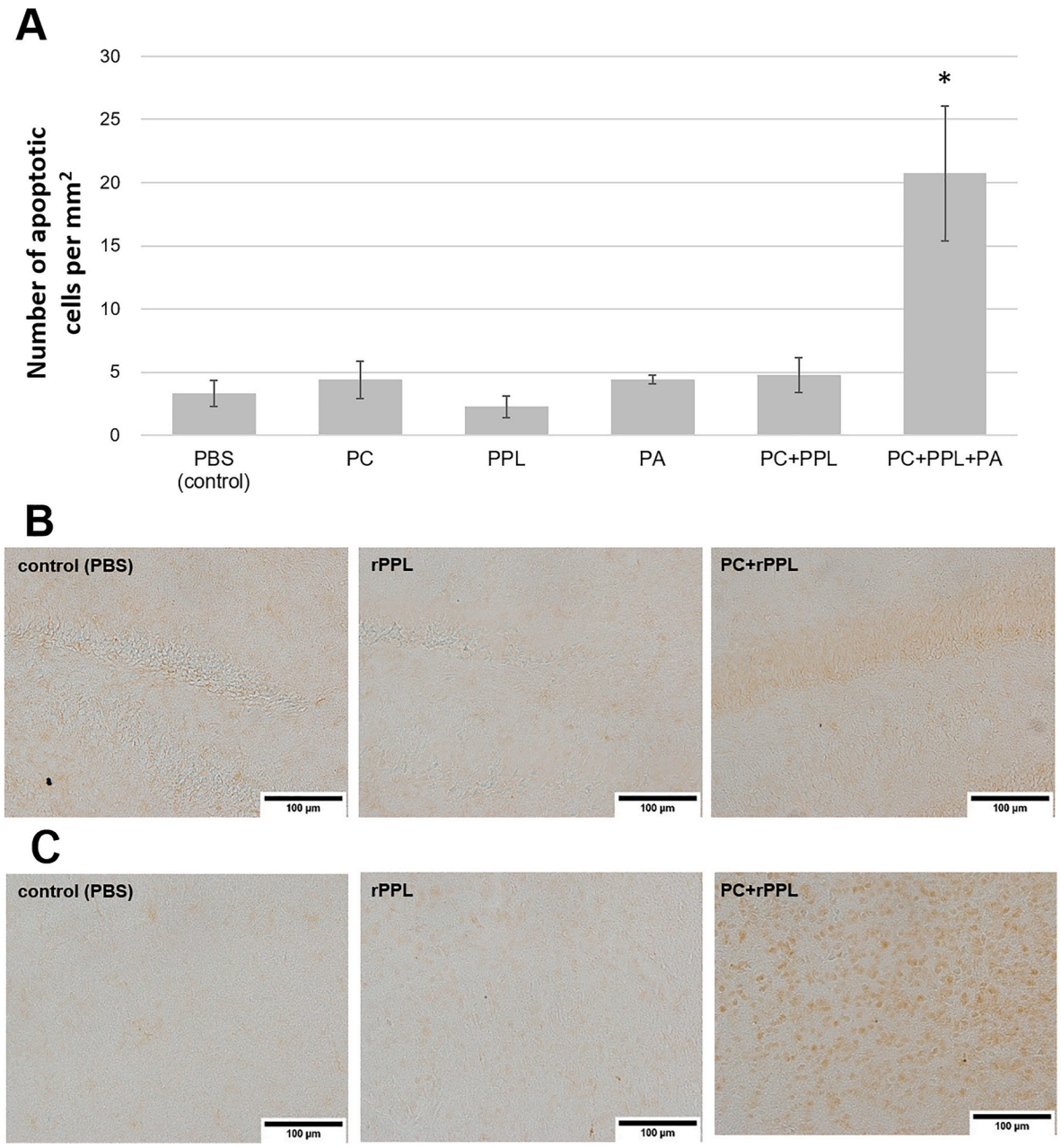

Fig. 4. Immunohistochemical analysis of the hippocampus of mice. (A) The number of apoptotic cells in the hippocampus of mice. Control: PBS, PC: PC (24 mg/kg), PPL: PPL (30 mg/kg), PA: PA (70 mg/kg), PC + PPL: PC (24 mg/kg) and PPL (30 mg/kg), PC + PPL + PA: PC (24 mg/kg), PPL (30 mg/kg) and PA (70 mg/kg). Values are mean ± SEM from 3 mice/group. Asterisk (*) indicates significant difference compared to control ($p < 0.05$). Detection of His-tagged rPPL in the hippocampus (**B**) and hypothalamus (**C**). Control: PBS, rPPL: rPPL (30 mg/kg), PC + rPPL: PC (24 mg/kg) and rPPL (30 mg/kg).

GFAP, and Glut1, which are brain biomarkers for mature neurons, dendrites, astrocytes, and endothelial cells, respectively, did not reveal any significant differences in the brains of mice intraperitoneally administered various combination of the samples (Fig. S7-S10). Taken together, the present study strongly suggests that the increase of apoptotic cells in the hippocampus was caused by the toxic effects of the compounds in combination. A more detailed analysis involving intracerebral administration, immunohistochemistry, and *in vivo* localization of the three substances is our next research target.

4. Conclusion

In a previous study, we successfully purified PC, a glycoprotein that exhibited lethal toxicity to mice, and demonstrated that the mixture of PC and PPL showed exo-protease activity, degrading substrates from their *N*- and *C*-termini with no amino acid specificity. Furthermore, when the mixture of PC, PPL, and PA was administrated to mice, apoptotic cell numbers were significantly increased in the hippocampus. The findings in this study will provide novel insights into the cases of acute encephalopathy attributed to this mushroom in 2004. To our knowledge, this is the first report of the involvement of three constituents in a food-poisoning outbreak resulting from consumption of a foodstuff.

Credit author statement

H.K. formulated the three-compound hypothesis and conceived the project. T.Su., F.M. and H.K. designed the experiment. T.Su., T.A., Y.T.,

L.N., T.Sa., Y.A. and J-H.C. performed the experiments. F.M., E.K., M.S., H.D., H.H., and H.K. assisted with experiments and contributed to discussions. T.Su. and H.K. wrote the manuscript with the help of E.K. and F.M. All authors critically reviewed the manuscript and approved the final version to be published.

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Ethical statement

All experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee of Utsunomiya university of Japan (Permission No. A17-0010) and performed in accordance with the approved guidelines and regulations. The authors compiled with the ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2022.106958.

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