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Fugu, *Takifugu ruberipes*, mucus keratins act as defense molecules against fungi

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ABSTRACT

Keratin is a cytoskeletal protein that constitutes the intermediate filament. Its distribution is restricted to epithelial tissues in mammals, but is wider in fish. An interesting feature of fish keratin is that it is abundant in the cutaneous mucus. However, the biological function of keratin in the mucus has not been explored. In the present study, we hypothesized that mucus keratins of fugu *Takifugu rubripes* function as antimicrobial molecules. To verify this hypothesis, we first identified all of the keratins expressed in the epidermis and present in mucus. Five of 15 keratins including Tr-K4 expressed in the epidermis were identified in the mucus. Subsequently, we examined the interaction of keratin molecules present in fugu mucus with yeast. Affinity chromatography using yeast as a carrier and subsequent LC–MS/MS analysis revealed that three types of keratin were bound to the yeast. Furthermore, yeast incubated with fugu mucus was agglutinated, and this was inhibited by anti-recombinant Tr-K4 (rTr-K4) antibody. Immunohistochemical analysis also revealed that keratin was attached to the surface of agglutinated yeasts. These findings indicate that mucus keratin agglutinates yeast. Furthermore, we found insoluble clumps in fugu mucus, which were mainly comprised of keratin. After incubation of yeast with soluble mucus fraction, insoluble clumps incorporating yeast were formed. This observation suggests that fugu mucus keratin sequesters microbes into insoluble clumps, which are eventually eliminated from the mucus. Here, we present our finding of the novel function of keratin as a defense molecule in fish mucus.

1. Introduction

Considerably more microorganisms inhabit the aquatic environment than land, therefore, skin mucus is a crucial fortification and defense for fish. Mucus is not only a physical barrier that obstructs the invasion of pathogens, but is also a chemical barrier that excludes them using defense molecules such as lectins, lysozymes, complement factors, transferrin, and immunoglobulin (Groff et al., 1997; Hatten et al., 2001; Schrock et al., 2001; Tsutsui et al., 2003; Rajan et al., 2011, 2013; Jurado et al., 2015).

Keratins are proteins that form intermediate filaments (IF) and are the most diverse molecules among cytoskeletal molecules. Keratins have been classified into two groups: type I and type II which are acidic and basic, respectively. A hetero-dimer is formed from one type I and one type II keratin molecule, which then forms a hetero-tetramer by binding to another dimer (Hatzfeld and Weber, 1990). Eight hetero-tetramers assemble into a unit-length filament (ULF) by lateral associations in cells (Herrmann et al., 2002). Following longitudinal annealing, ULFs assemble into keratin filaments, which form ropelike structures that confer mechanical properties of water-insolubility and resistance to tensile force.

Keratin molecules are remarkably diverse. In humans, 28 type I and 26 type II keratin genes have been identified to date. The distribution of keratins is confined to epithelial tissues in mammals. Keratins have specific expression patterns depending on the tissue type, for instance, cytokeratin 1 and 10 (K1/K10) are expressed in the suprabasal layer of skin, cytokeratin 3 and 12 (K3/K12) are distributed in the corneal epithelium, cytokeratin 4 and 13 (K4/K13) are localized in the esophageal mucous membrane, and cytokeratin 8 and 18 (K8/K18) are in the simple epithelium (Dale et al., 1985). The expression patterns of human keratins are strictly regulated.

In contrast to the keratin proteins in mammals, those in fish are not only distributed in epithelial tissues, but are also expressed in mesenchymal tissues comprised of cartilage or skeletal muscle cells (Groff et al., 1997; Conrad et al., 1998). Thus, tissue distribution of keratins is markedly complex in fish and a comprehensive elucidation of keratin distribution patterns has not been achieved in any fish species.

In addition to the abovementioned tissues, fish keratins are surprisingly also present in cutaneous mucus (Easy et al., 2009; Rajan et al., 2011; Jurado et al., 2015). Limited attention has been paid to the

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biological roles of mucus keratins probably because they have been regarded as residues of desquamated cells, and thus seem unlikely to serve any extracellular roles. However, Molle et al. (2008) reported that Tr65, a type II keratin isolated from skin mucus of the rainbow trout *Oncorhynchus mykiss*, possesses antibacterial activity against gram-negative bacteria, including *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Escherichia coli*, and gram-positive bacteria including *Staphylococcus aureus*, as a result of its pore-forming activity (Hatten et al., 2001). To date, this previous study is the only report to suggest that keratin protein plays a defensive role against microorganisms in skin mucus.

In the present study, we hypothesized that keratins act as defensive agents against microorganisms in fish skin mucus and examined this hypothesis in fugu *Takifugu ruberipes*. Since the marine environment contains various fungal as well as bacteria species (Das et al., 2006; Sogin et al., 2006) that fish need to defend themselves against, we used yeast *Saccharomyces cerevisiae* as a target microbes. First, we identified all keratin types in the mucus of fugu. We then demonstrated the binding and agglutinating activity of keratin to yeast. Furthermore, we found that mucus keratin forms insoluble clumps, which can entrap yeast. Herein, we present our finding of a novel function of keratin as a defensive agent in fugu cutaneous mucus. Our data provides further understanding of the functional features of keratin molecules and the defense system in fish skin mucus.

2. Materials and methods

2.1. Fish and sample preparation

Fugu samples were kindly supplied by Dr. Kikuchi of the Fisheries Laboratory, University of Tokyo or purchased from a fish-seller in Higashimurayama-City, Japan. Fugu were kept in a plastic tank with circulating filtered seawater under natural conditions.

Fugu skin mucus was collected by washing the body surface carefully with PBS using a syringe equipped with a needle to avoid artificial contamination of epidermal cells. The mucus was centrifuged (18,000 × *g*, 15 min), and separated into soluble and insoluble fractions, which were stored at -80 °C until use.

Untreated mucus was obtained by shaking the fugu gently in a plastic bag. The mucus was treated with 0.1% sodium azide and stored at 4 °C.

2.2. Gene expression analysis

Fish were killed with an overdose of 2-phenoxyethanol (Wako Pure Chemical Industries, Japan). The skin was peeled off and soaked in 2.4 units/mL dispase solution (Invitrogen, USA) at 25 °C with shaking. After 3 h, the epidermis was separated from the dermis by scraping the fish with the back side of a scalpel blade. Total RNA was isolated from the whole skin and the epidermal cells using an RNA extraction reagent (ISOGEN II, Nippon Gene, Japan). cDNA was synthesized using SMART™ RACE cDNA amplification kit (Clonthech, USA). Primers for fugu keratins found in the fugu genome database (http://asia.ensembl.org/index.html) were designed using Primer-Basic Local Alignment Search Tool (BLAST) (http://www.c¥ncbi.nlm.nih.gov/tools/prime-\r-blast/). All primer pairs were prepared to sandwich at least one intron. Each primer sequence is shown in Table 1. PCRs were conducted using the Advantage 2 Polymerase Mix (Clonthech) for krt1-19d (203–208), krt15 (1 of 2), krt15 (2 of 2), KRT80, krt222, and si dkey 222f2.1 (203–209) or Ex-Taq polymerase (TaKaRa Bio, Japan) for other genes. β-Actin was used as an internal control. PCR was carried out under the following conditions: 98 °C for 3 min, and 45 cycles at 98 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s.

2.3. 2D electrophoresis

Proteins from the soluble and insoluble fractions of the cutaneous mucus were subjected to two-dimensional gel electrophoresis (2D-PAGE). Each fraction was desalted using a PAGE-Clean Up kit (Nacalai Tesque, Japan), and the precipitates were dissolved in a rehydration buffer (8 M Urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate [CHAPS], 50 mM dithiothreitol [DTT], 0.2% BioLyte pH 3.0–10 (Bio-Rad, USA)). A ReadyStrip™ IPG strip (Bio-Rad) was rehydrated with rehydration buffer containing the mucus proteins. Isoelectric focusing was conducted using Ettan IPGphor 3 (GE Healthcare, Japan) under the following conditions: Step and hold, 300 V, 30 min, 0.2k Vhr; gradient, 1000 V, 30 min 0.3k Vhr; gradient, 5000 V, 80 min, 4.0k Vhr; and step and hold, 5000 V, 25 min, 2.0k Vhr.

The IPG strips were equilibrated with equilibration buffer 1 (50 mM Tris-HCl pH 8.8, 6 M Urea, 30% glycerol, 1% sodium dodecyl sulfate [SDS], and 0.25% DTT) and buffer 2 (50 mM Tris-HCl [pH 8.8], 6 M urea, 30% glycerol, 1% SDS, and 4.5% iodoacetamide) for 10 min, respectively. The strips were then subjected to PAGE using 12% polyacrylamide gels.

2.4. Western blotting

The mucus proteins separated using 2D-PAGE were blotted onto a polyvinylidene fluoride membrane. The blot was blocked with 0.5% skim milk (Wako Pure Chemical Industries) for 30 min, followed by washing with PBS plus 0.1% Tween20 (PBS-T) three times for 5 min each. The membrane was then incubated overnight at 4 °C in AE1/AE3 (PROGEN Biotechnik GmbH, BRD), an anti-human keratin mouse antibody cocktail that binds to the keratins in some fish, including trout, bichir, and sturgeons (Schaffeld and Markl, 2004). After washing the membrane with PBS-T three times for 5 min each, it was treated with horseradish peroxidase-conjugated anti-mouse IgG goat IgG (Sigma-Aldrich, USA) for 90 min. The enzyme reaction was then developed using a substrate solution containing 0.02 mg/mL 3, 3′-diaminobenzidine tetrahydrochloride and 0.006% hydrogen peroxide in 50 mM Tris-HCl (pH 7.5).

2.5. LC–MS/MS/MS

For LC–MS/MS analysis, the spots on the gel corresponding to those detected using western blotting were excised and digested with trypsin (Wako Pure Chemical Industries) at 37 °C for 20 h. Digested peptides were loaded onto an LC–MS/MS system consisting of an HPLC system (Nanospace SI-2; Shiseido Fine Chemicals, Japan) and an ion trap mass spectrometer (LCQ Deca; Thermo Finnigan, USA). Individual spectra obtained using the MS/MS system were processed using the Turbo SEQUEST software (Thermo Quest), and the generated peak list files were used for the SwissProt analysis using the Mascot program (http://www.matrixscience.com).

2.6. Binding assay of mucous keratins to yeast

The mucus soluble fraction was incubated with yeast at 4 °C overnight. After centrifugation (12,000 × *g*, 15 min), the supernatant was collected as the non-adsorption fraction. The pellet was washed with PBS four times, and the last washing buffer was recovered as the rinsing fraction. Subsequently, 8 M urea was added to the yeast pellet and incubated for 1 h at 4 °C. After centrifugation, the supernatant was recovered as the eluted fraction. These fractions were separated using SDS-PAGE and analyzed using western blotting with AE1/AE3. The bands on the gel corresponding to keratin detected in western blotting were excised and subjected to LC−MS/MS analysis as described above.

Table 1
Primer sequences of fugu, *Takifugu ruberipes*, keratins.

Type I Keratin	krt1-19d (201 and 202)	Forward	5′-GGATCTTCTGGCTATGCGCT-3
		Reverse	5′-TCTTCGGTTCTTGTTGGCGA-3′
	krt1-19d (203-208)	F	5′-GGACGACAACCTGATCGGAA-3′
		R	5′-CATGTTGGACACCTGAGCCT-3′
	krt15 (1 of 2)	F	5′-CAACCCGTGTCAATGGAGGA-3′
		R	5′-TGTAGTGTGCGACGGAGTTC-3′
	krt15 (2 of 2)	F	5′-CTTGGAGAGTAAGACCGCCC-3′
		R	5′-CCAGGTTCTGTAGTGTGCGA-3′
	zgc171226 (1 of 2)	F	5′-CATCCGGAGGCTTCAACCTT-3′
		R	5′-CCTTCATAGTGCTCGCGGAT-3′
	zgc171226 (2 of 2)	F	5′-GATTTCAAGATGAAGTTCGAGAACGAG-3′
		R	5′-TATTCCTGCTTGGTTTTGCTGATGTTG-3′
	si ch211-136j6.3	F	5′-GCGTAAGACAGTAGAGGCCG-3′
		R	5′-GCCTGAGTGATCTGGGTACG-3′
	zgc92380	F	5′-AAACTAGCTGAAGAAGACCTCAAAAACAAG-3′
		R	5′-GATACTCACATTTGGCGTTCAGTAG-3′
	KRT23	F	5′-CAAGAAGATCACCATGCAGAGTTTGAAC-3′
		R	5′-CTCTTTCTTCACATCAGTTGTCATGGTC-3′
	krt97	F	5′-GTCCGCCAACACAAAACTGG-3′
		R	5′-AAGGCTCTCTGTGCTTGACG-3′
	krt95 (201-203)	F	5′-ATGAGCAGTCGACAGTCTTGAG-3′
		R	5′-CACGTTGCCGATTTCTGTCTC-3′
	krt95 (204-206)	F	5′-TTTGACTTGTCCAGCGCCC-3′
		R	5′-TTCGTATTGCAGGCGGATCT-3′
	sich211-236k19.2 (204-210)	F	5′-AGAGTCCAGTACGAGGGCAT-3′
		R	5′-TTTCGACCTCCAGACCTTGC-3′
	lhx6 (2 of 2)	F	5′-GACGACTTCAGAATCAAGTGGGAGA-3′
		R	5′-CTCGTTGTTCTCCTTCACTTCGTTC-3′
	zgc77517	F	5′-GACGCTCCCAAAGGACAAGA-3′
		R	5′-TCCTCCAGGCGCATGATTAC-3′
	krt222	F	5′-ACCTCTCGATCCCGTGACTG-3′
		R	5′-CTTCATGGCGTCCGACTCTAA-3′
Type II Keratin	*zgc158846*	F	5′-GAAATCGACATGGTGAAATCCCAGAAAAA-3′
		R	5′-CATCTTCCTAAATCCTTCACTCTTCAAC-3′
	KRT80	F	5′-CATGGTGGGACTCAACGACA-3′
		R	5′-ACGCTGTCCTGCATTGAAGA-3′
	Tr-K4	F	5′-TGTTCGAGGCCTACATCAGC-3′
		R	5′-AGCCTTGGCTTCAAGTTCCA-3′
	krt8	F	5′-CCCGCTCAAACATTGATGCC-3′
		R	5′-TTGGCTTTGAAGCTCACGGA-3′
	si dkey222f2.1(201 and 202)	F	5′-GATATAGTCAACAATGAGAAGACGAAACTG-3′
		R	5′-GATTTGACAGCTTCTATCTCGGACTG-3′

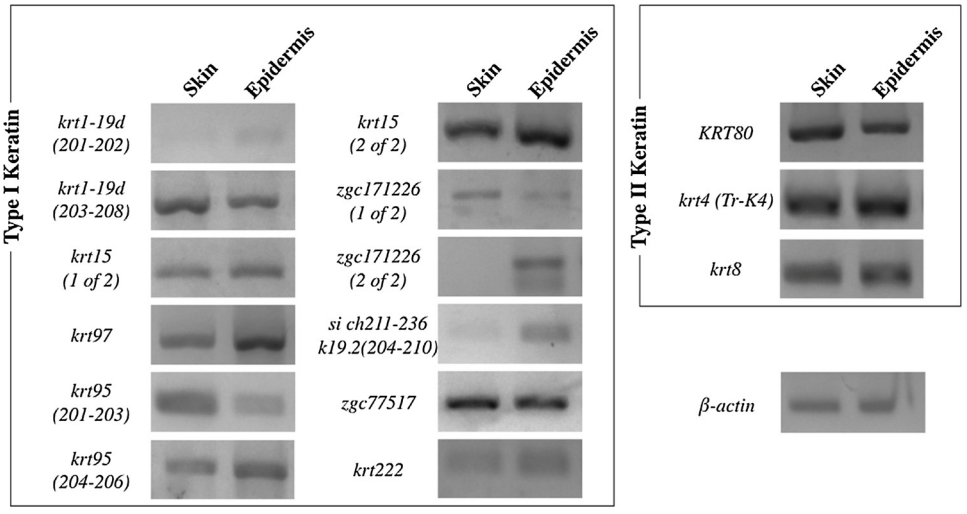

Fig. 1. RT-PCR analysis of keratin expression in fugu, *Takifugu rubripes*, epidermis. *β-Actin* was analyzed as the internal standard. The National Center for Biotechnology Information (NCBI) accession Nos. for each keratin are as follows: *Krt1-19d (201–202)*: XM011604355, *krt1-19d (203–208)*: XM003965032, *krt15 (1 of 2)*: XM011617712, *krt97*: XM003972130, *krt95 (201–203)*: XM003972497, *krt95 (204–206)*: XM003972498, *krt15 (2 of 2)*: XM003979544, *zgc171226 (1 of 2)*: XM011621151, *zgc171226 (2 of 2)*: XM011612333, *si ch211-236 k19.2 (204–210)*: XM003975209, *zgc77517*: XM011614273, *krt222*: XM011612561, *KRT80*: XM003966565, *Tr-K4*: XM003973624 and *krt8*: XM003963019.

2.7. Preparation of rTr-K4

Eighty-two amino acid residues from ^{6}Ser to ^{88}Ala of fugu K4 (Tr-K4), a type II keratin (National Center for Biotechnology information [NCBI] accession No: XP003973673), was cloned using a primer pair for Tr-K4 including a restriction enzyme site, fugu skin cDNA template and Ex-taq polymerase, and the amplicon was ligated into pGEM T-Easy vector (Promega, USA). The Tr-K4 sequence of the pGEM T-Easy were digested at restriction enzyme sites using Nco 1 and Xho1, which recognize and digest the CCATGG and CTCGAG sequences, respectively, and ligated into pET32b vector (Novagen, USA) that was predigested with the same enzymes. The plasmid was introduced into *E. coli*

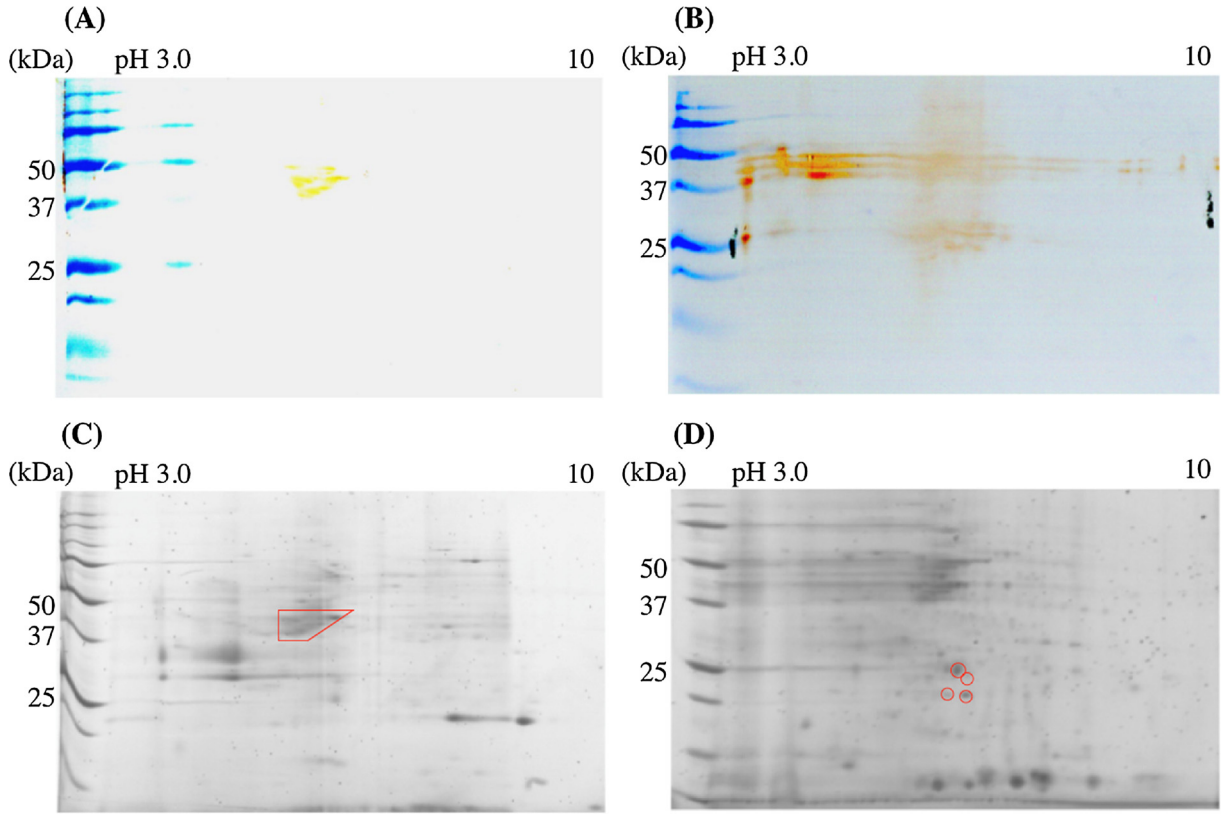

Fig. 2. Western blotting map (A and B) using an anti-human keratin antibodies cocktail (AE1/AE3) and Coomassie Brilliant Blue (CBB) stained map (C and D) of two-dimensional electrophoresis gel of mucus extraction as soluble (A and C) and insoluble (B and D) fractions from fugu cutaneous mucus. In the soluble fraction keratin-positive signals were detected in the area between 37 and 50 kDa (A). We excised the area indicated by the line from the CBB stained gel (C) corresponding to the keratin protein spots in (A) for the LC–MS/MS analysis. In the insoluble fraction, keratin-positive signals were detected in the area at 25 kDa and between 37 and 50 kDa (B). Four spots indicated by the red circle were excised from the area at 25 kDa (D) corresponding to keratin protein spots in (B) for the LC–MS/MS analysis.

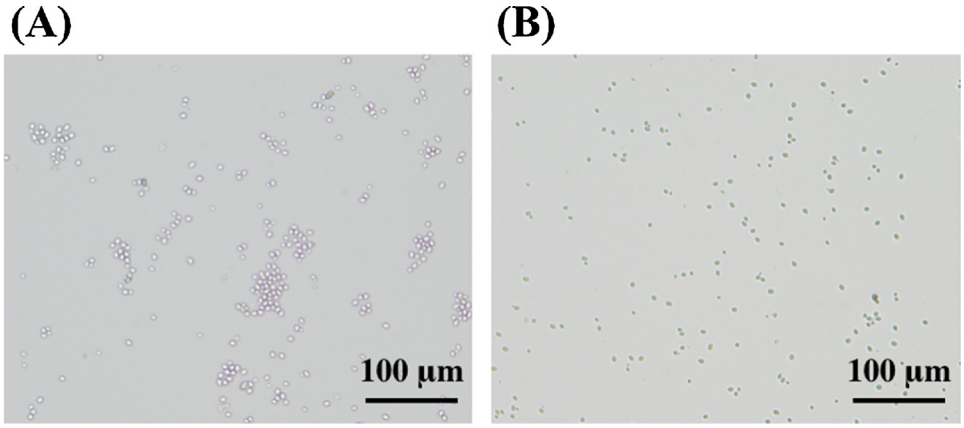

Fig. 3. Microscopy of agglutinated yeast. Yeast incubated with the soluble fraction of fugu cutaneous mucus (A). Control yeast samples were incubated with PBS (B).

BL21(DE3)pLysS single competent cells (Novagen). The transformants were cultured in 500 mL Lysogeny broth (LB) medium containing 20 mg/mL of ampicillin and 0.1 M IPTG for 3 h at 37 °C. The bacterial cells were suspended in PBS and sonicated using an ultrasonic cell disruptor apparatus (UR-21 P, TOMY). Following centrifugation, the supernatant was applied to a Ni Sepharose™ 6 fast flow column (GE Healthcare). After incubation for 30 min at 4 °C, the column was washed with 50 mM imidazole in PBS and then the rTr-K4 was eluted with 0.5 M imidazole in PBS.

2.8. Preparation of the anti-rTr-K4 polyclonal antibody

One milligram rTr-K4 was used to raise the rabbit polyclonal antibody. The rTr-K4 was emulsified with Freund complete and incomplete adjuvants and injected into the rabbit four times to generate anti-rTr-K4 serum by ARK-Resource Co., Ltd. Pre-immune serum was obtained from the same rabbit before immunization. The IgG fraction including anti-rTr-K4 IgG was obtained from the rabbit antiserum using Ab-Rapid SPIN™ 10 (ProteNova, Japan).

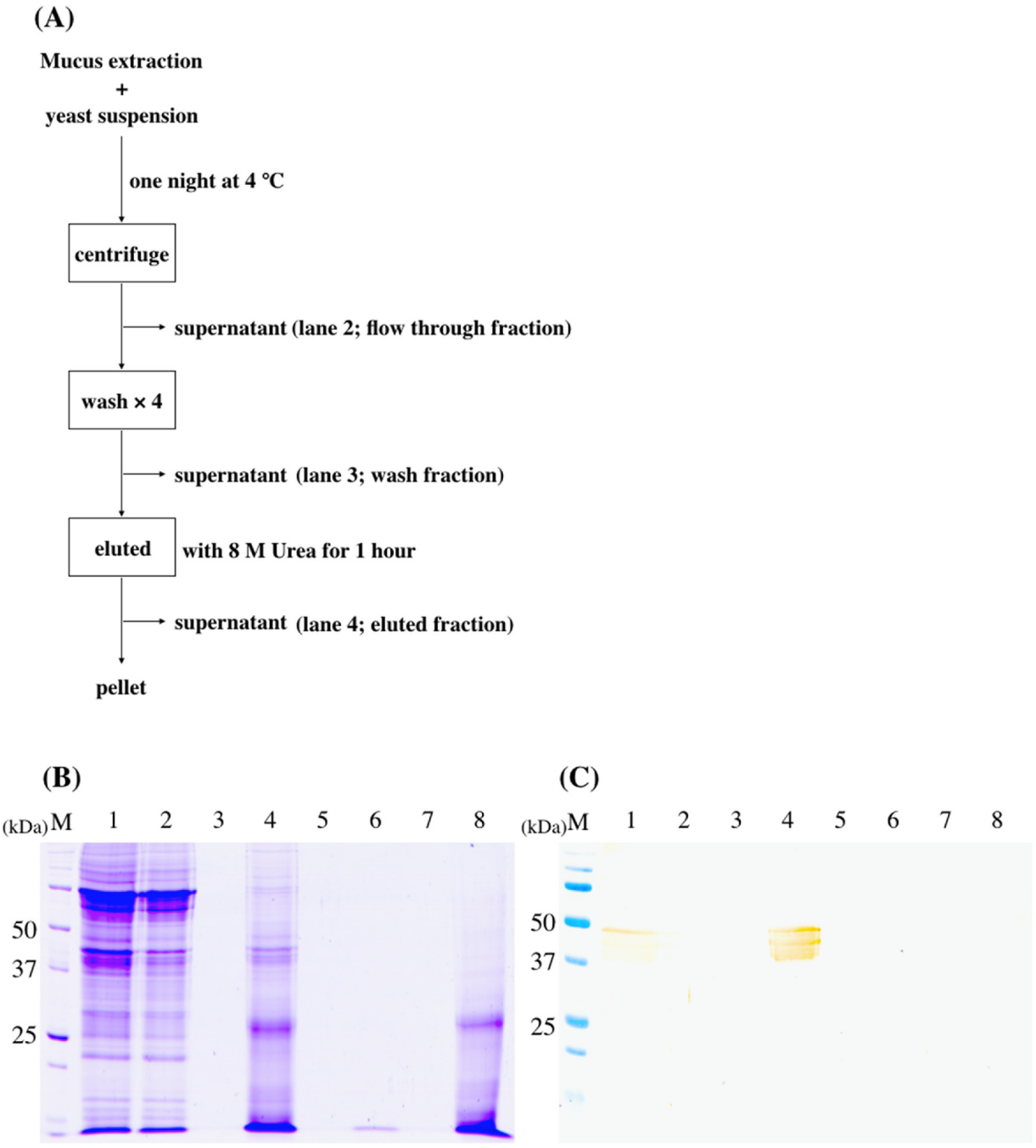

Fig. 4. Process flow chart of affinity chromatography using yeast as the carrier (A). SDS-PAGE of yeast binding protein eluted with 8 M urea from fugu cutaneous mucus (B). First, mucus extract (lane 1; untreated mucus) was mixed with yeast suspension for 1 night at 4 °C. The supernatant after centrifugation was regarded as a flow through fraction (lane 2), and the pellet was washed with PBS four times. The last wash buffer was regarded as the wash fraction (lane 3). Yeast-binding protein was eluted with 8 M urea for 1 h at 4 °C. After centrifugation, the supernatant was collected (lane 4). For the negative control, PBS was substituted for the mucus extract (lanes 5–8). (C) Western blotting of fractions using AE1/AE3 antibody.

2.9. Confirmation of antibody specificity

The purified rTr-K4 and soluble fraction of fugu mucus were subjected to 2D-PAGE and western blotting using the anti-rTr-K4 IgG to confirm the specificity of our antibody. Then, the detected spots were then analyzed using LC-TOF/MS as described below. The peptides from the keratin spot were subjected to the BLAST analysis and Tr-K4 sequence alignment.

2.10. LC-TOF/MS analysis

The gel spots in the 2D-PAGE of the fugu mucus corresponding to the membrane spots detected using anti-rTr-K4 IgG in the western blot analysis, were excised and subjected to LC-TOF/MS analysis. In brief, the excised gel spots were subjected to reductive alkylation with 1 µL of the 1 M DTT and 100 µL of 50 mM sodium bicarbonate (NH_4CO_3) for 10 min at 80 °C, and cooled with ice. Then, 2 µL 1 M iodoacetamide was added and incubated for 15 min at 37 °C. For decoloration of the gel, 100 µL each of 100% methanol and 10% acetic acid was added to the gel sediment. After vortexing for 10 min, the supernatant was removed,

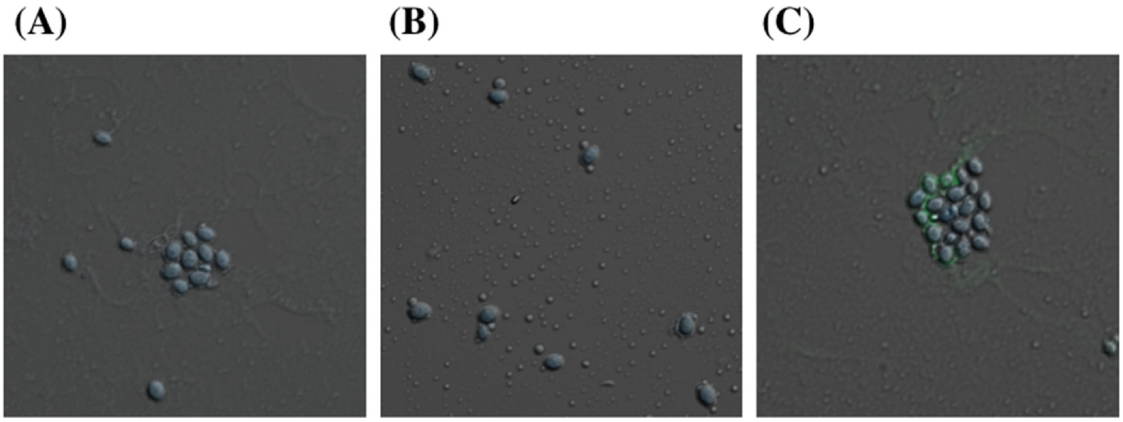

Fig. 5. Immunohistochemical analysis of yeast agglutinated by fugu mucus using AE1/AE3 and Alexa488-conjugated secondary antibodies (C). Keratin signals were detected on the agglutinated yeast. The negative control for immunohistochemical analysis showed that no signals were detected (A). Without mucus, yeast agglutination and keratin signal were not observed (B).

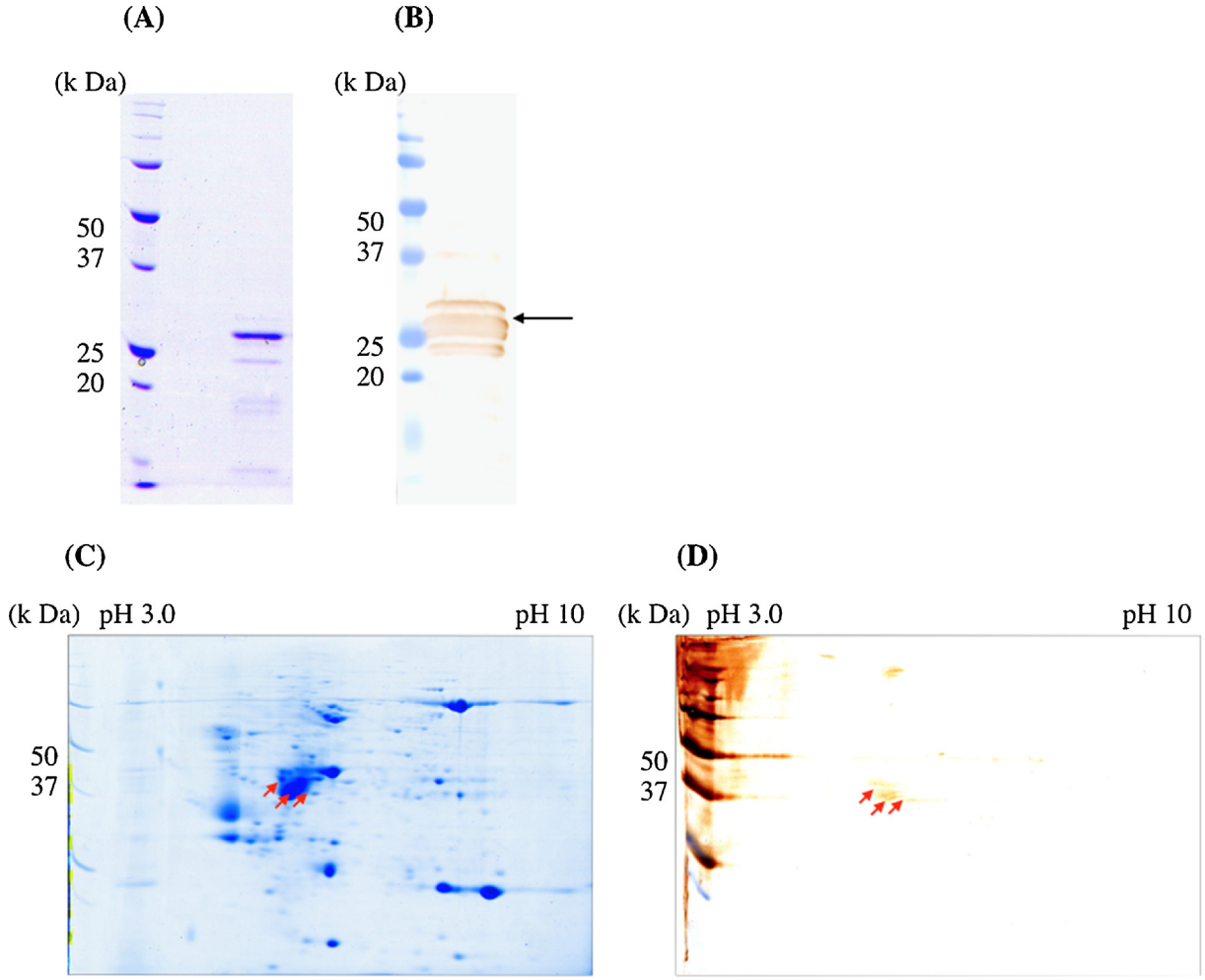

Fig. 6. Confirmation of specificity of anti-rTr-K4 antibody. (A and B): SDS-PAGE of protein affinity-purified from transformants using a Ni-sepharose column, followed by Coomassie Brilliant Blue (CBB) staining (A) or western blotting with anti-rTr-K4 antibody (B). One major band at 27 kDa was seen in (A) and a corresponding band (arrow) was seen in (B). (C) and (D): 2D electrophoresis of the fugu mucus soluble fraction followed by CBB-staining (C) or western blotting with anti-rTr-K4 antibody (D). (D) Two spots at 40 kDa were detected by the antibody. Corresponding spots on the gel were subjected to LC-TOF/MS analysis. Spots are indicated by arrows in (C) and (D).

the pellet was washed with 100 µL of 100% acetonitrile, and the proteins in the gel were then digested with 0.05 mg/mL trypsin and incubated overnight at 37 °C. The digested peptides were extracted using an ultrasonic vibration sieving machine, and 10 µL each of 0.2% formic acid and 100% acetonitrile were added. The sample was subjected to a Triple TOF® 5600+ system (SCIEX, Japan) and the peptide sequences obtained using this system combined with the analysis software Peakview (SCIEX) were subjected to BLAST analysis to identify the

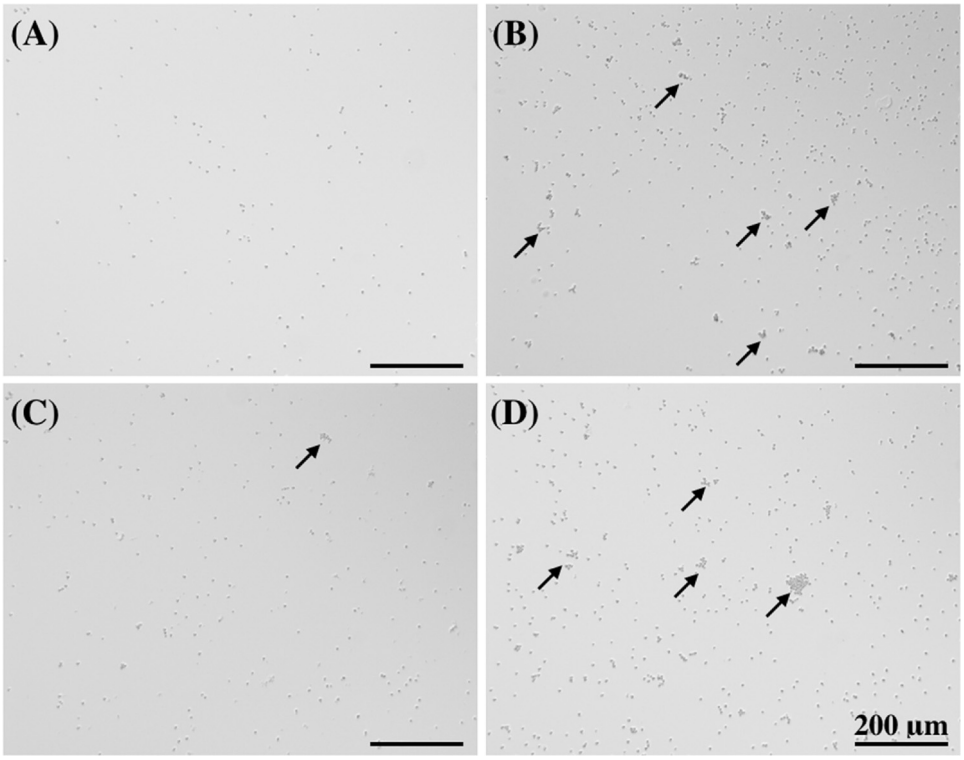

Fig. 7. Inhibition of yeast agglutination by anti-rTr-K4 rabbit IgG revealed by differential interference microscopy. Yeast samples were incubated with PBS (A), fugu mucus (B), fugu mucus pre-incubated with anti-rTr-K4 IgG (C), or fugu mucus-pre-incubated with pre-immunization rabbit IgG (D). Arrows indicate agglutinated cells.

protein.

2.11. Inhibition of yeast agglutination using anti-rTr-K4 antibody

The soluble mucus fraction was incubated with anti-rTr-K4 IgG overnight at 4 °C. For the control, IgG purified from pre-immunization rabbit serum was substituted for anti-rTr-K4. The yeast suspension was added to each fraction and incubated overnight at 4 °C. After incubation, 5 µL of each sample was dropped onto a glass slide and observed microscopically.

2.12. Immunofluorescence microscopy

The soluble fraction was incubated with fixed yeast overnight. The mixture was then dropped onto a glass slide and dried at room temperature. After washing with PBS, the slides were blocked with 10% normal goat serum for 30 min at room temperature. Subsequently, the specimens were washed with PBS and incubated with AE1/AE3 for 90 min. After washing, Alexa 488-conjugated anti-mouse IgG (Abcam, UK) was added onto each specimen, and incubated for 90 min in at room temperature. After washing, the specimens were mounted with DAPI-Fluoromount-GTM (Southernbiotech, USA) and observed using a confocal microscope LSM800 (Carl Zeiss, BRD).

2.13. Characterization of insoluble clumps in fugu cutaneous mucus

Untreated mucus was filtered using a cell strainer (70 µm; FALCON, Japan) and separate into insoluble clumps and other proteins. The obtained clumps were washed with PBS and dissolved with 1% SDS. Subsequently, the solution was subjected to SDS-PAGE and western blotting using an AE1/AE3 antibody. The bands in the gels corresponding to those detected by AE1/AE3 in the western blotting, were excised and subjected to LC-TOF/MS to identify the proteins as described above. Moreover, we detected the keratin signal of the clumps incorporated with yeast using immunofluorescence with the AE1/AE3 antibody as described above.

3. Results

3.1. Keratin genes expressed in the epidermis

We identified 21 keratin genes in the fugu genome database. Among these, 15 keratin genes, including 12 and 3 type I and II keratins, respectively were expressed in the epidermis of fugu based on RT-PCR analysis (Fig. 1).

3.2. Keratin in the cutaneous mucus

To identify the keratin molecules present in the cutaneous mucus, we performed 2D-PAGE for soluble and insoluble fractions of mucus, followed by western blotting and LC-MS/MS analysis. Several spots were detected using the AE1/AE3 antibody cocktail in the range of 37–50 kDa in both the soluble and insoluble fractions in the western blot (Fig. 2). A few of the 25 kDa spots detected in the insoluble fraction were not found in the soluble fraction. In the LC-MS/MS analysis, several peptides matching five keratin protein sequences, including Tr-K4, krt97, krt1-19d (203–208), krt15 (2 of 2), and krt95 (201–203), were detected in an area containing multiple spots in the soluble fraction gel (Fig. 2), and some peptides matching Tr-K4 were detected in one of four spots at 25 kDa in the insoluble fraction gel (Fig. 2 and Table 3). This result showed that at least five keratin proteins were present in fugu cutaneous mucus. Tr-K4, detected in both the soluble and insoluble fractions, was the only type II keratin identified in the mucus.

3.3. Mucus keratins bound to yeast

Yeasts were agglutinated after overnight incubation with the mucus (Fig. 3). To determine the yeast-agglutinin in the mucus, proteins that bound to the yeast were eluted with 8 M urea and analyzed using SDS-PAGE. The results showed that the eluted fraction contained multiple bands of 37–50 and 25 kDa (Fig. 4B). Since the 25 kDa bands were also seen in the control fraction, they were considered to be derived from the yeast. In the western blotting using AE1/AE3 antibody cocktail to

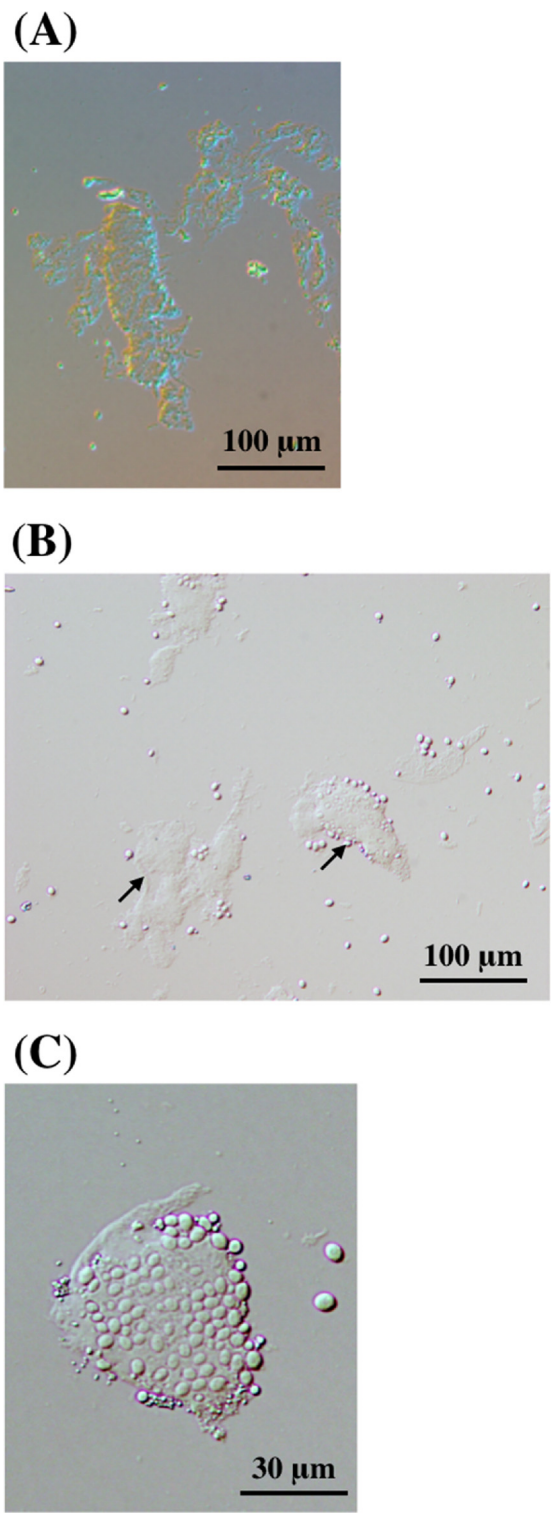

Fig. 8. Insoluble clumps in fugu mucus revealed by differential interference observation. (A) Untreated mucus. (B) Soluble fraction of mucus after overnight incubation with yeast. Clumps incorporating many yeast cells were seen (arrows). (C) Higher magnification.

keratins, the 37–50 kDa bands were also detected in the eluates (Fig. 4C), suggesting that fugu mucus keratins bound to the yeast. LC–MS/MS analysis revealed that the three bands corresponding to the positive bands in the western blotting using AE1/AE3 were fugu keratin proteins. A MASCOT search identified krt1-19d (203–208) and Tr-K4 in the upper band, only krt1-19d (203–208) in the middle band, and Tr-K4

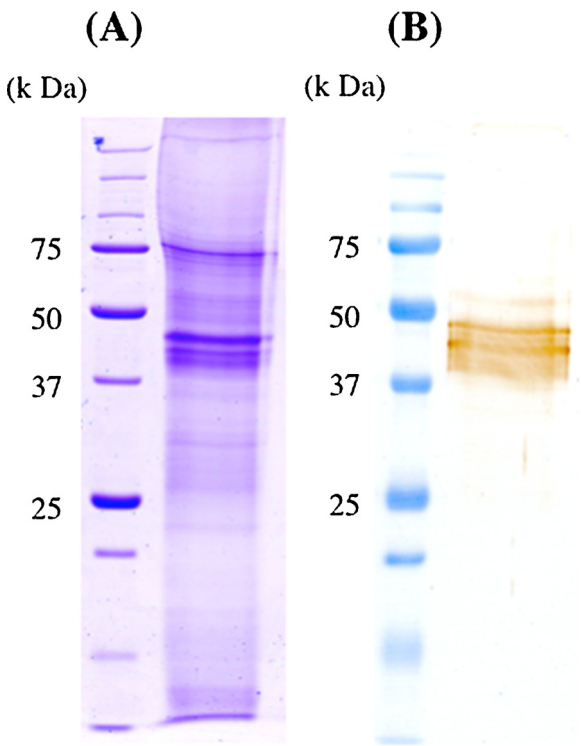

Fig. 9. SDS-PAGE of insoluble clumps followed by CBB staining (A) or western blotting using AE1/AE3 antibody (B).

and krt15 (2 of 2) in the lower band (Table 3). For further confirmation, immunofluorescence analysis using AE1/AE3 was performed. Positive signals of keratins were clearly detected at the surface of the aggregated yeasts (Fig. 5). These data indicated that fugu mucus keratins were bound to and agglutinated the yeast.

3.4. Preparation of recombinant Tr-K4 (rTr-K4) and anti-rTr-K4 antibodies

First, we prepared recombinant Tr-K4 (rTr-K4) to immunize rabbits to raise the anti-rTr-K4 antibody. To prepare the recombinant protein, we selected 82 residues from the ^{6}Ser to ^{88}Ala of Tr-K4, which showed lower identities to other fugu keratins. As shown in Fig. 6, the synthesis of rTr-K4 was induced by 0.1 M isopropyl β-D-thiogalactopyranoside (IPTG). After purification of the rTr-K4 using a Ni-column and imidazole, the major 26 kDa band was observed in the SDS-PAGE gel (Fig. 6A). The band was consistent with the expected molecular mass of rTr-K4, which contains a large 17 kDa tag, indicating that the protein was purified successfully. We then immunized the rabbit with the purified rTr-K4, and obtained the antiserum, which was subjected to Ab-Rapid SPIN™10 to collect the IgG fraction. In the western blotting, the antibody detected rTr-K4 in the purified rTr-K4 fraction (Fig. 6B). To confirm the specificity of the antibody, we performed western blotting for fugu mucus and LC-TOF/MS analysis. Three spots were detected (Fig. 6C and D). Several peptide sequences were obtained from two spots, and no peptides sequences were obtained from the remaining spot. The BLAST search showed that the analyzed peptides from the spots matched the Tr-K4 sequence but not those of other keratins. These data indicated that the antibody recognized Tr-K4 with high specificity.

3.5. Tr-K4 agglutinates yeast

To examine whether Tr-K4 agglutinates yeast, we performed an inhibition assay using purified anti-Tr-K4 antibody. As shown in Fig. 7, anti-rTr-K4 antibody clearly inhibited agglutination of the yeast while the IgG from normal rabbit serum did not affect yeast agglutination.

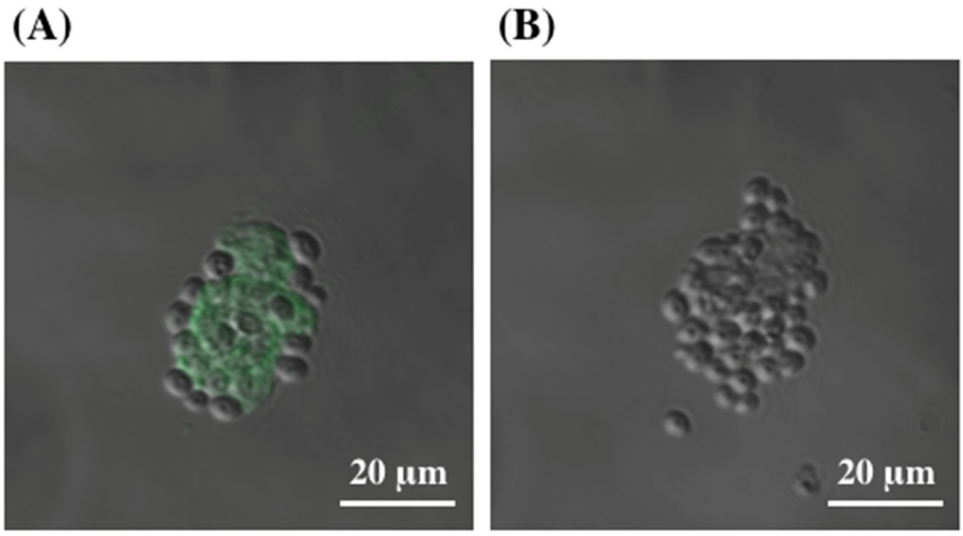

Fig. 10. Immunohistochemical observation of clumps incorporating yeast cells revealed by differential interference observation. (A) AE1/AE3 and Alexa488-conjugated secondary antibody staining. (B) PBS substituted for primary antibody.

Table 2
Primer sequences used for the preparation of recombinant Tr-K4.

rTr-K4	Forward	5′-CCATGGACTCTGTCAGAGAATACAGCAC-3′
	Reverse	5′-CTCGAGAGCGATCATGGCTGCATTGGAG-3′

Table 3
Protein hits using the Mascot search in the LC–MS/MS analysis of the keratins detected in the soluble and insoluble fractions of fugu, *Takifugu rubripes*, mucus.

	Keratin	Score	Accession No.
Fugu keratins identified from mucus			
Soluble fraction	Tr-K4	550	XP-003973673
	krt97	98	XP-003972179
	krt1-19d (203-208)	490	XP-003965081
	krt15 (2 of 2)	424	XP-003979593
	krt95 (201-203)	118	XP-003972546
Insoluble fraction	Tr-K4	53	XP-003973673
Fugu keratins identified from yeast binding fraction			
Upper band	krt1-19d (203-208)	283	XP-003965081
	Tr-K4	209	XP-003973673
Middle band	krt1-19d (203-208)	80	XP-003965081
Lower band	Tr-K4	226	XP-003973673
	krt15 (2 of 2)	222	XP-003979593

These findings indicated that Tr-K4 in the cutaneous mucus is at least one of the required keratin molecules for yeast agglutination.

3.6. Keratins form insoluble clumps in cutaneous mucus

Microscopic observation revealed that the untreated fugu mucus contained insoluble and irregular-shaped clumps (Fig. 8A). When the soluble mucus fraction was incubated with yeast in a microtube rotating overnight, yeast agglutination occurred. Furthermore, insoluble clumps incorporating yeast were found (Fig. 8B, C). To identify the molecules forming these clumps, we separated the clumps using a cell strainer and subjected them to SDS-PAGE. The clumps were found to be composed of multiple proteins, including 37–50 kDa proteins, as the major components (Fig. 9A). These proteins were positively stained by AE1/AE3 in the western blotting (Fig. 9B). Moreover, LC-TOF/MS analysis identified these proteins as Tr-K4, krt1-19d (203–208), and krt15 (2 of 2). These data indicated that the major components of the insoluble clumps in fugu cutaneous mucus were keratin proteins.

Immunofluorescence staining using AE1/AE3 and Alxa488-conjugated secondary antibodies, also showed that the insoluble clumps that contained yeasts were composed of keratin (Fig. 10). Collectively, these observations suggested that soluble keratin molecules bound to yeast and were accumulated to form insoluble clumps.

4. Discussion

Although fish mucus contains numerous keratins, their roles in the mucus have hardly been investigated (Molle et al., 2008). In the present study, we demonstrated that keratins in the fugu cutaneous mucus bound to yeast and agglutinate them. Furthermore, keratins in the mucus formed insoluble clumps, into which the yeast were incorporated and embedded. These findings demonstrated that fugu cutaneous mucous keratins act as defense molecules via their agglutinating activity and also by uniquely excluding microorganisms by incorporating them into insoluble clumps.

Using 2D-PAGE and MS analysis,five keratin types were identified in fugu mucus, of which krt1-19d (203–208), Tr-K4, and krt15 (2 of 2) were identified in the yeast-bound fraction. However, we did not clarify which keratins bound directly to the yeast. Type I and II keratins form heterodimers, which further polymerizes into a filamentous structure in cells. If two types of keratin molecules also assemble in the mucus, the only type II keratin found in the mucus, Tr-K4, may play a key role in binding yeast. We could not exclude the possibility that other types of keratins were present in the mucus; however, it is likely that these three keratins were the major yeast-binding proteins. Further studies are needed to clarify whether these keratin proteins are polymerized in fugu cutaneous mucus should be clarified.

The estimated molecular mass of Tr-K4, based on its amino acid sequence, is 53 kDa. However, we detected Tr-K4 in the 25 kDa spot in the insoluble fraction of mucus and 37 kDa bands of the soluble mucus fraction (Fig. 2). The MS analysis indicated that the 25 kDa protein was the C-terminus of Tr-K4. Human cytokeratin 6-derived peptide in corneal epithelial cells has an antimicrobial function (Tam et al., 2012); therefore, fragmented fugu mucus keratins may also serve specific biological roles.

Proteomic studies have revealed that the cutaneous mucus of fish includes cytoskeletal molecules such as actin and keratins (Rajan et al.,

2011; Cordero et al., 2015; Jurado et al., 2015; Sanahuja and Ibarz, 2015). However, to date, a comprehensive analysis of keratins in fish has not yet been conducted. The present study, for the first time, identified keratins distributed in the epidermis and mucus of fugu. Our data showed that 15 keratin genes were expressed in the epidermis (Fig. 1), including at least 5 that were present in the cutaneous mucus (Table 2). We also analyzed the expression of keratins in other several other tissues including other mucosal tissues, such as the intestines and gills, and non-mucosal tissues, such as the kidney, liver, and muscle. Interestingly, *Tr-K4* was also expressed in the liver and kidney (not data shown). Thus, Tr-K4 is not specific to mucosal tissues, although it is unknown whether it plays any additional roles other than forming cytoskeleton in those tissues.

We found insoluble clumps in fugu cutaneous mucus, which were mainly composed of some keratin species. Furthermore, insoluble clumps incorporating many yeast cells were formed in the soluble fraction of mucus after incubation with yeast. If the same process occurrs in fish skin, microorganisms trapped in the clumps would be excluded by mucus shedding. Therefore, this finding presents a possible novel function of keratins as defense molecules in fish mucus. Although the mechanisms by which the yeast-embedding clumps were formed is unknown, it is plausible that fugu keratin molecules bound to the yeast polymerize to insoluble forms on the cell surface. Further studies are needed to elucidate the molecular mechanism of clump formation and how it may differ from that of yeast-agglutination.

It is unknown whether the mucus keratins are released into the mucus through a secretory pathway from epidermal cells or just following cell death. Fugu keratins have no signal peptide; however, in *Anopheles gambiae*, actin, a structural molecule lacking a signal peptide, is secreted extracellularly and the secretion is regulated by the Imd and Toll pathways (Sandiford et al., 2015). In vitro experiments are needed to clarify the source of mucus keratin, i.e., to verify whether keratin is secreted from cells will be needed.

Another question to be answered is how fugu keratin molecules bind to yeast. Although the interaction of keratins with the yeast could be induced by ionic factors, there is presently no supporting evidence. Thus, future studies needed to clarify the binding mode of keratin to yeast. Specifically, the state of keratin molecules, such as monomers, dimers, tetramers, or larger that can interact with yeast and the specific site in keratin molecules responsible for the binding require clarification.

In conclusion, we demonstrated that fugu mucus keratins act as defense molecules. They not only bind to microorganisms, causing agglutination but also form clumps to incorporate and exclude offending pathogens. These findings suggest that keratin plays a pivotal role in the antimicrobial barrier of fish skin mucus. Future studies are needed to further our understanding of the properties of keratins beyond simply existing as protein component of the cytoskeleton.

Declaration of Competing Interest

None.

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