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Original Article

Type-I-hypersensitivity to 15 kDa, 28 kDa and 54 kDa proteins in vitellogenin specific to *Gadus chalcogrammus* roeKeiko Hanaoka^a, Shunsuke Takahagi^a, Kaori Ishii^a, Miyako Nakano^b, Yuko Chinuki^c, Akio Tanaka^a, Yuhki Yanase^a, Michihiro Hide^{a,*}^a Department of Dermatology, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan^b Unit of Biotechnology, Graduate School of Integrated Sciences for Life, Hiroshima University, Hiroshima, Japan^c Department of Dermatology, Shimane University Faculty of Medicine, Shimane, Japan

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ABSTRACT

Background: Fish roe allergy is a common health problem in countries where sea food is a major part of the diet, such as Japan. β' -component (β' -c) in fish roe has been identified as a major antigen for patients who show hypersensitivity to various fish roes. However, little is known about causative antigens for patients reactive to fish roe of specific species.

Methods: Serum and basophils were obtained from patients who had reactivity to roes of *Gadus chalcogrammus* (GC) and/or other fish species. GC roe specific antigens were analyzed by immunoblotting, histamine release assay (HRA) and mass spectrometry. Recombinant-fragments of vitellogenin (Vg) were obtained by the *Escherichia coli* expression system.

Results: Serum IgE of a patient with specific reactions to GC roe bound to 15, 28, 40 and 70 kDa-proteins in GC roe extract. Mass spectrometry analysis revealed that proteins in these bands contained fragments corresponding to Vg. Immunoblotting of Vg immunoprecipitated by rabbit anti-Vg antiserum from the extract revealed 15, 28 and 54 kDa fragments bound by the patient's IgE. These bindings were inhibited by the pretreatment of recombinant phosvitin (rPv) and β' -c (r β' -c). Fractions obtained by native gel electrophoresis containing 15, 28 and 54 kDa proteins, but not the other fractions, induced significant histamine release from the patient's basophils. Sera of the other patients with GC roe specific-IgE showed IgE binding to rPv and/or r β' -c.

Conclusions: The 15, 28 and 54 kDa-fragments of Vg which include structures of Pv and β' -c, could be antigens for GC roe specific type-I-hypersensitivity.

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Introduction

The number of patients with food allergy has been increasing in the world. Among various causes of food allergy, fish roe is one of the major antigens especially in children and is the culprit in 3.7% of the cases.¹ Oral intake of fish roe including specific antigens leads to IgE-mediated hypersensitivity. The antigen activates mast cells and/or basophils by bridging the antigen-specific IgE bound to the high affinity IgE receptors (Fc ϵ RI) on the cells. The activated cells release several inflammatory mediators, such as histamine which consequently leads to individuals developing clinical symptoms.

Patients with fish roe allergy present with wheals, respiratory symptoms and/or gastrointestinal symptoms, possibly resulting in life-threatening anaphylactic reactions due to oral intake of causative fish roes. The preference and consumption of fish roe depends on geographical and cultural conditions. It is a common and indispensable foodstuff in many countries, such as Japan. Thus, the identification and analysis of antigens in fish roe is required to develop precise diagnostic measures and new treatment, such as prophylaxis, for fish roe allergy.

The analysis of the antigen in patients with salmon roe allergy revealed that the major antigen in fish roe is β' -component (β' -c), a degraded fragment of vitellogenin (Vg) and a constituent of the oocyte in fish ovaries.² Vg is a precursor protein synthesized in fish liver which is then conveyed to the oocyte through the blood circulation.³ Accumulated Vg in fish oocytes is proteolytically degraded to the three major yolk proteins; lipovitellin (Lv), β' -c and phosvitin (Pv) (Supplementary Fig. 1).⁴ β' -c shares highly

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homogeneous amino acid sequences with immunological cross-reactivity among teleost fish roes.⁵ Salmon and rainbow trout, both of which belong to Salmonidae, share 95% homogeneity of the amino acid sequence of their β' -cs.² Moreover, salmon in Salmonidae and walleye pollock in Gadidae share 60% of sequence homology in the N-terminal 20 residues in β' -c.⁵ Indeed, specific IgE against β' -c in sera of many patients with salmon roe allergy have been shown to be cross-reactive to β' -c of other fish such as salmonid fish, walleye pollock, flatfish and shishamo smelt.^{2,4,6} Thus, patients with fish roe allergy may respond to β' -c of a wide range of fish roes with cross-reactivities.^{6,7} However, a certain population of patients show the hypersensitivity only to a specific species of fish roe. In such patients, culprit antigens that induce allergic reactions have not been identified and their immunoreactivities to β' -c or other yolk proteins remain unknown. In this study, we analyzed the fish roe antigen that caused allergic reaction in a patient, who showed specific reactivity only to *Gadus chalcogrammus* (GC) roe, by immunoblotting and using histamine release assay (HRA) with the use of GC roe extract, immunologically-purified Vg and recombinant Vg fragments.

Methods

Patients with fish roe allergy

The diagnosis of fish roe allergy was made by allergy specialists, based on the patients' clinical history and clinical investigations, such as skin prick test, HRA and fish roe-specific IgE determination in sera. Skin prick tests were performed by "prick-to-prick" fashion using material from fish roes in their natural, original state. Fish roe-specific IgE titers were measured by the ImmunoCAP methodTM (Thermo Fisher Scientific/Phadia AB, Uppsala, Sweden). HRA was performed as described below. This study was approved by the institutional review boards of ethics in Hiroshima University Hospital (approval number, E-1179) and Shimane University Hospital (approval number, 469). Informed consents were obtained from all participants.

Proteins and total RNA extraction from the liver and roe of fish

The liver of a female GC, and roes of GC and other fish were prepared for protein and total RNA extraction. Two kinds of GC roe

samples were used for experiments in this study. One was GC roe already processed at a store, and the other was harvested from a female GC at our laboratory. The former was used for experiments shown in Figure 1 and 2, and the latter was analyzed in experiment in Figure 3–5. Proteins were extracted from homogenized tissue samples into RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) by sonication. After centrifugation, the supernatants were collected as the extracts of GC roe and other fish roes, respectively. Total RNAs were extracted from the liver and roe of GC according to the manufacturer's instructions of RNeasy-ICE Frozen Tissue Transition Solution (Invitrogen, Carlsbad, CA, USA) and ISOGEN (Nippon Gene, Tokyo, Japan).

Protein extraction from native page gel

Protein was eluted from native-PAGE gel slice in a cellulose membrane tube with a 3.5kD size pore (Spectra/Por, Spectrum Laboratories, Rancho Dominguez, CA, USA) filled with Tris-glycine buffer by electrophoresis. The solvent of the recovered protein was replaced with PBS by dialysis.

Plasmid construction and expression of recombinant proteins

Total RNA obtained from GC roe converted to cDNA with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). cDNA fragments of Pv and β' -c of GC roe were amplified by PrimeSTAR HS DNA Polymerase (Takara Bio, Shiga, Japan) and specific primer pairs for Pv and β' -c (Pv sense primer with incorporated XhoI digested fragment, 5'-GCCGCTCGAGAACGCCAGGGCAG; Pv antisense primer with PstI digested fragment, 5'-GCCGCTGCAGCAACGGGTACACAA-CAGTG; β' -c sense primer with XhoI digested fragment, 5'-GCCGCTCGAGATCATGGAAAAAGTCCAATACGTC; β' -c antisense primer with PstI digested fragment, 5'-GGCGGCTGCAGAGCACA-GAGGTTGGC). The DNA fragments were double-digested with XhoI and PstI, then ligated into pCold TF DNA (Takara Bio). For bacterial expression of pCold TF DNA by using *Escherichia* (*E.*) *coli*, BL21 (Takara Bio), cultures from the clone of BL21 that contained constructed plasmid vectors were lysed with xTractor buffer kit (Takara Bio), and the polyhistidine tagged- and TF-fused recombinant Pv (rPv) and β' -c (r β' -c) were extracted by HisTARON gravity columns purification kit (Takara Bio), according to the manufacturer's instructions.

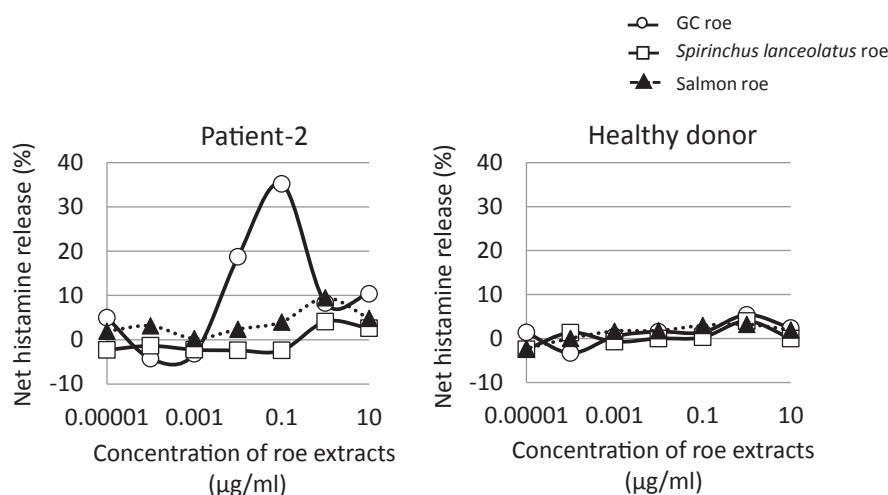


Fig. 1. Histamine release assay (HRA) against fish roe extracts at indicated concentrations. *Gadus chalcogrammus* (GC) roe extract induced 35.2% histamine release from basophils of Patient-2 at the concentration of 0.1 μ g/ml, but neither salmon roe nor *spirinchus lanceolatus* roe induced significant release of histamine. None of GC, salmon roe and *spirinchus lanceolatus* roe extracts induced histamine release from basophils of a healthy donor.

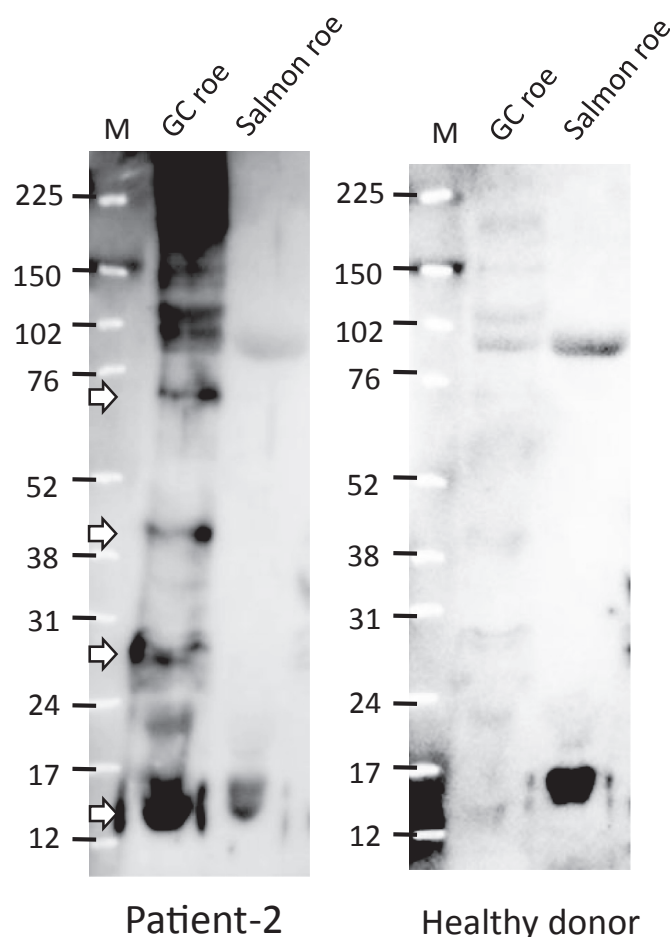


Fig. 2. Immunoblotting of fish roe extracts with serum of Patient-2. Serum of Patient-2 revealed specific IgE binding to 15, 28, 40 and 70 kDa-proteins (arrows) in *Gadus chalcogrammus* (GC) roe extract. Several proteins larger than 100 kDa were detected by IgE of Patient-2. However, the same size, although somewhat faint, proteins were detected by IgE of the healthy control as well. Sera of both Patient-2 and a healthy donor showed bindings of their IgE to 100 and 16 kDa proteins of salmon roe.

Protein dephosphorylation assay

GC roe extract (100 µg/ml) was mixed with 120,000 U/ml Lambda phosphatase (SANTA CRUZ Biotechnology, Dallas, Texas, USA) and 0.2 mM MnCl₂, and incubated at 30 °C overnight. The reaction was stopped by incubating at 65 °C in the presence of 50 mM EDTA for 5 min. Protein dephosphorylation was detected by immunoblotting with anti-phosphoserine antibody (Abcam Plc, Cambridge, UK).

SDS-PAGE and immunoblotting

The denatured or native protein samples were loaded into 5–20% SDS-PAGE gel or 15% Native-PAGE gel (ATTO, Tokyo, Japan) and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) using a semi-dry blotting system (ATTO). After blocking by Blocking one (Nakalai tesque, Inc., Kyoto, Japan) for 1 h, the membrane was incubated with the patient's serum diluted with sample buffer, (TOYOBO, Tokyo, Japan) (1:100) or antibody (1:10000) at 4 °C overnight. After washing five times with tris-buffered saline, membrane was incubated with horse radish peroxidase (HRP)-conjugated goat anti-human IgE (KPL, Gaithersburg, MD, USA) or HRP-conjugated goat anti-rabbit IgG (Cell

Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. After washing with tris-buffered saline, chemiluminescence were detected. In the competitive assay, the patient's serum was pre-incubated with indicated fish roe extracts.

Immunoprecipitation of Vg

The GC roe extract was incubated with rabbit anti-Vg antisera (Abnoba, Taipei, Taiwan) and Protein A-Sepharose beads (Sigma—Aldrich Japan, Tokyo, Japan), followed by centrifugation and collecting the pellet. The pellet was denatured with sample buffer including dithiothreitol (DTT) under heat to remove Protein A-Sepharose beads. After centrifugation, the supernatant composed of the mixture of Vg and anti-Vg antibody was collected.

HRA

Peripheral blood leucocytes containing about 1% basophils were obtained from patients or healthy volunteers and subjected to HRA as previously described.^{8,9} Briefly, basophilic leukocytes were stimulated with indicated concentrations of fish roe lysates and 0.3 µg/ml of goat anti-human IgE antibody (Bethyl Laboratories, Montgomery, TX, USA). The amounts of released histamine were measured by the high performance liquid chromatography (HPLC) system consisted of an LC-solution (Shimadzu, Kyoto, Japan) as previously described.⁸

Results

The reactivity to fish roes in patients with fish roe allergy

We first investigated the reactivity to fish roes in three patients with fish roe allergy. Patient-1 had clinical history of oral and pharyngeal swelling with gastrointestinal symptoms in response to both GC roe and Salmon roe. Patient-2 and Patient-3 had histories of oral and pharyngeal swelling against GC roe or salmon roe, respectively (Table 1). Both skin prick tests and the amount of specific IgE against each fish roe revealed sensitivities of the patients as suggested by their clinical histories. We then investigated the biological activity of GC roe and other roes on the basophils of Patient-2. HRA revealed 35.2% histamine release in response to GC roe extract from the basophils of the patient, but neither salmon roe nor *Spirinchus lanceolatus* roe induced significant release of histamine (Fig. 1). None of the roe extracts induced histamine release from basophils of a healthy donor. Since the antigen of GC roe specific allergy has not been previously reported, we hereafter analyzed the specific antigen in GC roe by using serum obtained from Patient-2.

Detection of specific antigen of GC roe by means of immunoblotting and mass spectrometry

We investigated if specific proteins in GC or salmon roe lysate are bound to IgE in serum of Patient-2. Lysates of GC roe and salmon roe were prepared for SDS-PAGE, and western blotting was performed with serum of Patient-2. Several proteins of GC roe, but none of salmon roe, were specifically bound by serum IgE of Patient-2 at positions of 15, 28, 40 and 70 kDa (Fig. 2). Mass spectrometry analysis (Supplementary Methods) revealed that proteins recovered from these four bands contained fragments corresponding to Vg, which has two variant proteins, Vg-A and Vg-B (Supplementary Figs. 1, 2, 3 and Supplementary Methods). The ratio of Vg-A and Vg-B fragments to all detected peptides was 18% and 13% in 15 kDa band, 9% and 13% in 28 kDa

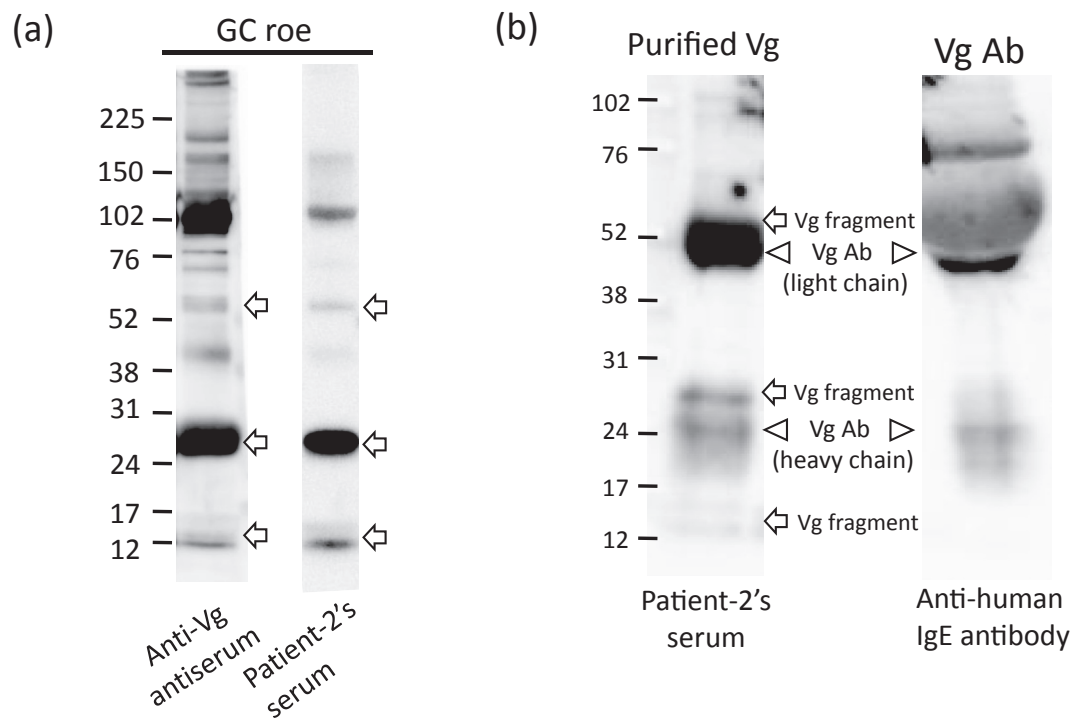


Fig. 3. (a) Immunoblotting of *Gadus chalcogrammus* (GC) roe extract with polyclonal anti-vitellogenin (Vg) antiserum. Rabbit anti-Vg serum detected various proteins including 15, 28, 40, 54 and 70 kDa-bands (arrows) in GC roe extracts which were bound by IgE in serum of Patient-2. (b) Immunoblotting of Vg immunoprecipitated with rabbit anti-Vg sera from the extract of GC roe. The 15, 28 and 54 kDa-Vg fragments (arrows) were detected by serum IgE of Patient-2. The right panel shows that the anti-Vg serum (Vg Ab) contained 24 and 45 kDa (arrowheads) proteins, presumably light and heavy chains of rabbit immunoglobulin, which react with anti-human IgE antibody. Thus, blottings at the same sizes in the left panel are likely due to the binding of anti-human IgE to immunoglobulins bound to Protein-A and intermingled in the sample.

band, 36% and 25% in 40 kDa band, and 23% and 12% in 70kDa band, respectively.

The reactivity of patient's serum to purified Vg fragments

Immunoblotting of the GC roe extract with polyclonal anti-Vg antiserum detected multiple proteins including 15, 28, 40, 54 and 70 kDa proteins corresponding to those detected by serum IgE of Patient-2 (Fig. 3a). In order to confirm that Patient-2's IgE binds to Vg, we isolated Vg from GC roe by means of immunoprecipitation by rabbit anti-Vg antiserum and performed immunoblotting with serum of Patient-2. As shown in Figure 3b, the 15 kDa, 28 kDa and 54 kDa fragments immunoprecipitated by anti-Vg antiserum were detected by serum IgE of Patient-2 as well.

Specific IgE binding and histamine release activities of 15 kDa, 28 kDa and 54 kDa fragments

We then performed competitive immunoblotting of the GC roe extracts with serum of Patient-2. As shown in Figure 4a, the bindings of Patient-2's IgE to 15, 28 and 54 kDa fragments in the GC roe extract were abolished by the pre-treatment of the serum with GC roe extract, while only a slight inhibition was observed by the pre-treatment with salmon roe and *spirinchus lanceolatus* roe. These results indicate that Patient-2's IgE specifically binds to the proteins in GC roe extract.

Native-PAGE and subsequent analysis of the fractionated proteins were performed in order to evaluate histamine release activity of the GC roe fragments. The GC roe extract was separated into 4 fractions by native-PAGE (Fig. 4b). The 15, 28 and 54 kDa proteins were detected by serum IgE of Patient-2 in the denatured immunoblotting in fraction (Fr) #3 and #4, respectively (Fig. 4c). As

shown in Figure 4d, Fr#3 and Fr#4 induced 39.9% and 38.4% histamine release, respectively at most from basophils of the Patient-2, but not from basophils of a healthy control. Thus, the 15, 28 and/or 54 kDa proteins could induce IgE-mediated histamine release from the basophils of Patient-2 with GC toe allergy.

Immunological analysis of the 15 kDa, 28 kDa and 54 kDa fragments by using rPv and rβ'-c

Pv in Vg-A of GC showed the lowest homologous amino acid sequence among fish roes, implying an immunogenicity of Pv in the allergy specific to GC roe (Supplementary Fig. 4). Since Pv harbors the most abundant phosphorylated sites of serines in Vg fragment, we then investigated the presence of phosphoserine in Vg fragments and its involvement with the binding to IgE. As shown in Figure 5a, all of the serum IgE of Patient-2, anti-Vg antiserum and anti-phosphoserine antibody detected 28 kDa protein in GC roe. Moreover, both the molecular weight and the amount of binding by the antibodies was decreased by the treatment of dephosphorylation (Fig. 5a). These results suggest that the 28 kDa protein has several phosphorylation sites required for the binding of IgE in serum of Patient-2.

We examined if Pv in Vg-A is recognized by IgE in serum of Patient-2 and analyzed its immunoreactivities to the common fish roe antigen, β'-c, which shows cross-reactivity to various kinds of fish roes. cDNA coding rPv and rβ'-c were expressed in *E.coli* as described in the materials and methods section. Since the triggering factor (TF) protein (52 kDa) derived from the vector were added to each protein, the expected molecular weights of rPv and rβ'-c were 68 and 80 kDa, respectively. Immunoblotting of the GC roe extract, rPv and rβ'-c showed that serum IgE of Patient-2 bound to 68 kDa rPv and 80 kDa rβ'-c tagged by polyhistidine (Fig. 5b) as

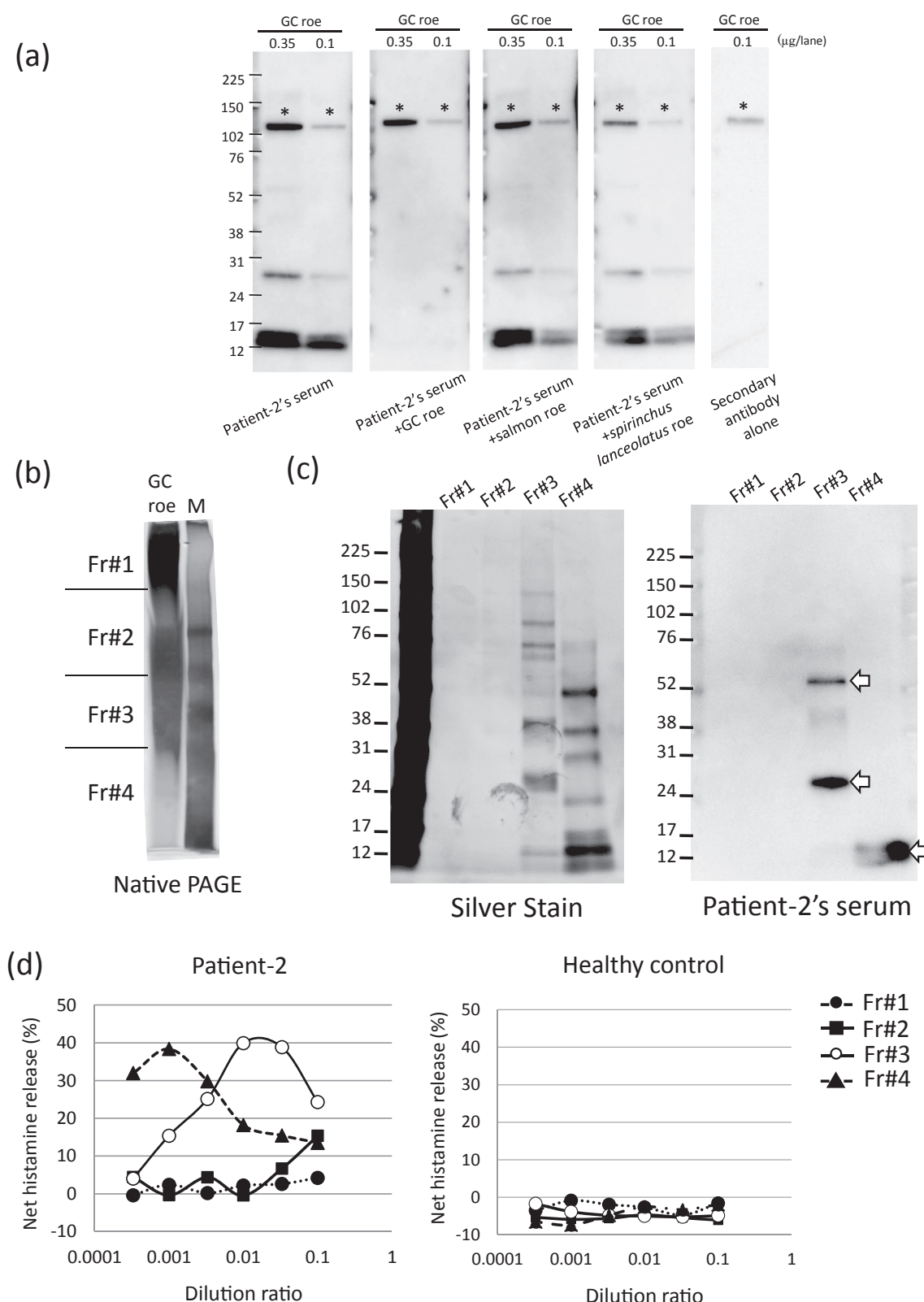


Fig. 4. (a) Competitive immunoblotting of the *Gadus chalcogrammus* (GC) roe extracts with the serum of Patient-2 pretreated with fish roe extracts. The binding of IgE antibody to the GC roe extract was abolished by the pre-treatment of the serum by the GC roe extract, while only little inhibition was observed by the salmon roe extract and the *Spirinchus lanceolatus* roe extract. The band at the position of 102 kDa (*) was the non-specific binding of the secondary anti-human IgE. (b) Native-PAGE of the GC roe extract and its fractionation. The GC roe loaded under the native condition was separated and purified into four fractions (Fr). (c) Protein staining and immunoblotting by Patient-2's serum of the fractionated GC roe extracts electrophoresed under the reduced conditions in SDS (Fr#1, Fr#2, Fr#3 and Fr#4). The 28 kDa, 54 kDa and 15 kDa proteins reactive to serum IgE of Patient-2 were detected in Fr#3 and Fr#4 (arrows). (d) Histamine release assay (HRA) against the fractionated GC roe extracted. Both Fr#3 containing 28 kDa and 54 kDa, and Fr#4 containing 15 kDa proteins, but none of the other fractions, induced significant histamine release from basophils of the patient.

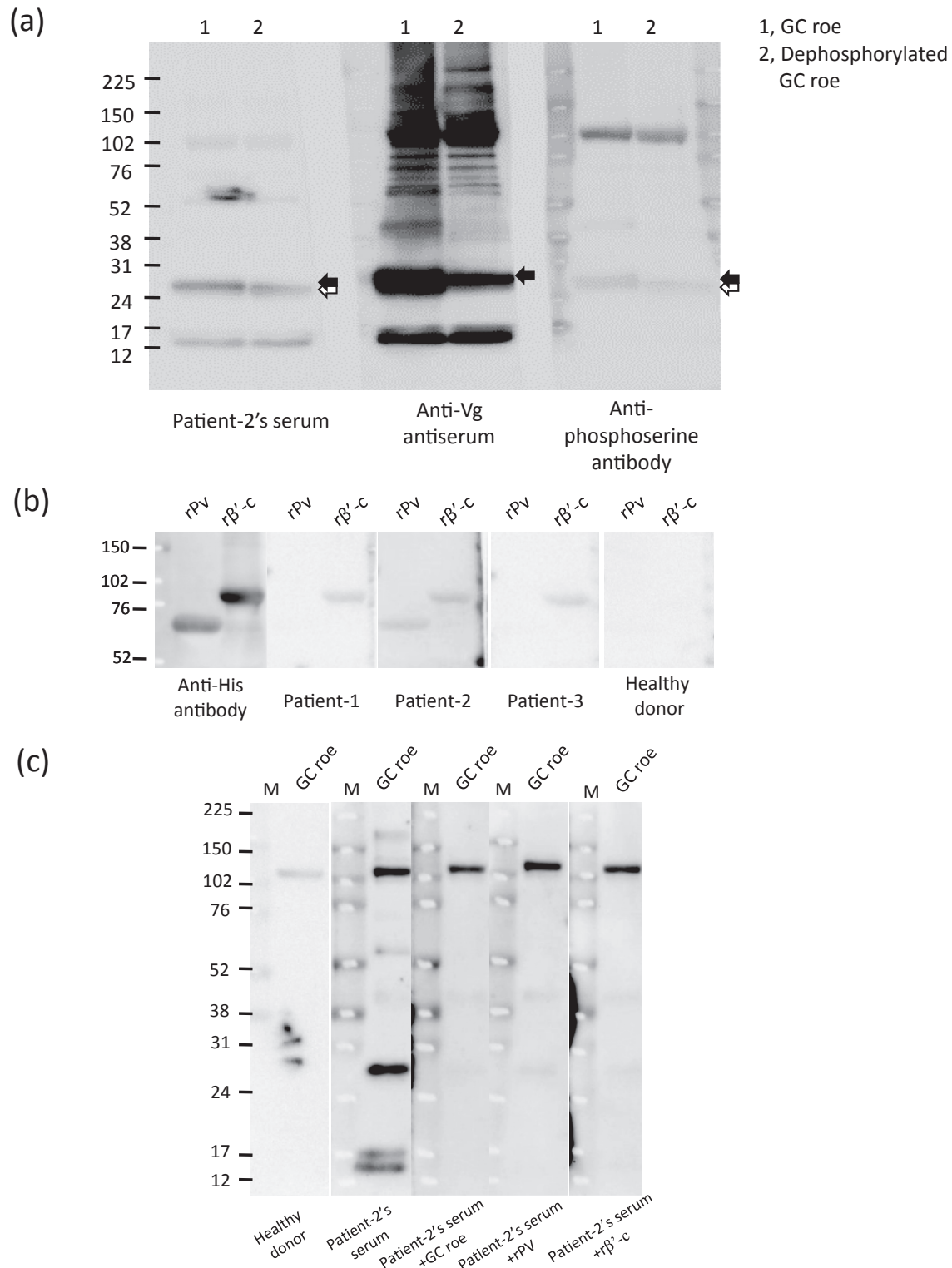


Fig. 5. (a) Protein dephosphorylation assay of *Gadus chalcogrammus* (GC) roe extracts. The anti-Vg antiserum detected the decreased amount of 28 kDa proteins by the dephosphorylation treatment (black arrow). The serum of Patient-2 and the anti-phosphoserine antibody detected the reduced amount and molecular weight of 28 kDa protein (black arrows) to 25 kDa (white arrows). (b) Immunoblotting of recombinant phosphitin (rPv) and β' -component (r β' -c) with sera of Patient-1-3. Both Patient-1 and Patient-3 showed IgE binding only to r β' -c, but IgE of Patient-2 bound to both rPv and r β' -c tagged by polyhistidine at the position of 68 kDa and 80 kDa. (c) Competitive immunoblotting of the *Gadus chalcogrammus* (GC) roe extract with the serum of Patient-2 pretreated with rPv and r β' -c. The binding of the Patient-2's IgE to both 15 and 28 kDa proteins was inhibited by the pretreatment of rPv or r β' -c as well as by the GC roe extract.

Table 1

Clinical characteristics of patients with fish roe allergy.

Patient	Age	Sex	Diagnosis	Clinical symptoms	Skin prick test		Specific IgE (UA/ml)	
					GC roe	Salmon roe	GC roe	Salmon roe
Patient-1	7	male	GC roe and salmon roe allergy	Oral and pharyngeal swelling with gastrointestinal symptoms	Positive	Positive	14.6	13.7
Patient-2	22	female	GC roe allergy	Oral and pharyngeal swelling	Positive	Negative	23	<0.1
Patient-3	16	male	Salmon roe allergy	Oral and pharyngeal swelling	Negative	Positive	0.71	5.79

well as 15, 28 and 54 kDa proteins in the GC roe extract (Fig. 5c). The binding of IgE to 15, 28 and 54 kDa proteins in GC roe extract was reduced by the pre-treatment of the serum with either rPv or rβ'-c (Fig. 5c), suggesting that the antigens in GC roe to the patient's IgE are 15, 28 and 54 kDa proteins including structures of Pv and β'-c in Vg. Nevertheless, neither 68 nor 80 kDa recombinant protein induced histamine release from basophils of Patient-2 (data not shown).

Finally, we analyzed the relation between the clinical reactivity specific to GC roe and IgE-binding profile to rPv and rβ'-c among three clinically well-characterized patients, Patients-1, -2, and -3. Both Patient-1 and Patient-3 showed IgE binding only to rβ'-c, but IgE of Patient-2 bound to both rPv and rβ'-c (Fig. 5b). Clinically, Patient-1 was reactive to both GC roe and non-GC fish roe, whereas Patient-3 was reactive only to non-GC fish roes, and Patient-2 was specifically sensitive to GC roe (Table 1). Thus, IgE binding to fish roe β'-c caused cross-reactivity to GC roe and a non-GC roe, or reactivity to a non-GC roe, but IgE binding to Pv was more specific to GC roe than that to β'-c. These results suggest that 15 kDa, 28 kDa and 54 kDa proteins contains the partial structures of Pv and β'-c, and that the biologically specific hypersensitivity to GC roe in Patient-2 might be developed by the sensitization with epitope(s) in Pv.

Binding ability of IgE to rPv and rβ'-c detected in sera with GC roe specific IgE

We examined the binding of serum IgE to rPv and rβ'-c with sera obtained from other four patients (Patient-4-7), in which GC roe-specific IgE (>0.35 UA/ml) was detected by ImmunoCAP™. As shown in Figure 6, IgE-binding to rPv was detected in Patient-4 and -5, and that to both rPv and rβ'-c in Patient-7, whereas Patient-6 showed no IgE-binding to both recombinant proteins. This result suggests that there is several patterns of IgE binding to rPv and rβ'-c found in sera

with GC roe specific IgE, and not only β'-c, a well-known fish roe antigen, but also Pv can be an IgE reactive antigen in GC roe as indicated in the analysis by using the serum of Patient-2.

Discussion

In this study, we demonstrated 15 kDa, 28 kDa and 54 kDa proteins which include structures of Pv and β'-c as the antigens for a patient hypersensitive specifically against GC roe. Although patients with fish roe allergy tend to show cross-reactivity to various kinds of proteins in fish roes, certain patients with fish roe allergy may be sensitized to proteins specific in a particular kind of fish roe, such as GC roe.

Among the fragments of Vg, β'-c has been known as a major antigen of fish roe allergy with high cross-reactivity among fish roes. It consists of two subunits of 16 kDa and 18 kDa proteins sharing extremely similar amino acid sequence and IgE reactivity.¹⁰ The prediction of its secondary structure based on an amino acid sequence indicates that 1/2 of the whole β'-c constitutes the beta sheet structure lacking the formation of the alpha helix structure.¹⁰ This structure of the high thermostable and resistant to enzyme digestion make β'-c a stable antigen in fish roe. Lv light chain was also reported as another antigen in fish roe allergy,⁵ and a study suggested a fragment of Vg including both β'-c and Lv as an antigen in anaphylactic cases of salmon roe allergy.⁶ On the other hand, Pv, another fragment of Vg, has been considered to have a low potential of IgE sensitization and reactivity because Pv includes 51 residues of serine which is expected to undergo much phosphorylation.¹¹ However, our patient in this study had specific IgE against Vg fragments containing structures of Pv as well as that of β'-c. Moreover, blotting shown in Figure 5a suggests a crucial role of phosphorylation in the antigenicity of 28 kDa protein against IgE in serum of Patient-2. Further investigation by using several sera obtained from patients with positive GC roe specific IgE titer

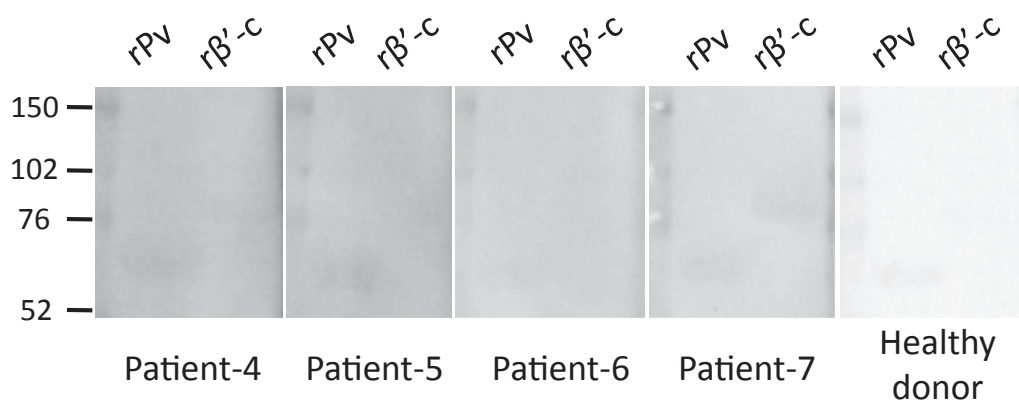


Fig. 6. Immunoblotting of rPv and rβ'-c by sera with *Gadus chalcogrammus* (GC) roe specific IgE. The binding of IgE to rPv and rβ'-c was examined by sera of four patients (Patient-4-7), in which GC roe-specific IgE (>0.35 UA/ml) was detected by ImmunoCAP™. IgE-binding to rPv was detected in Patient-4 and -5, and that to both rPv and rβ'-c in Patient-7, but Patient-6 showed no IgE-binding to both recombinant proteins.

revealed the IgE binding to rPv in 3 out of 4 patients, indicating that Pv can be an antigen for IgE.

In this study, 15 kDa, 28 kDa and 54 kDa proteins immunologically detected by serum IgE of Patient-2 were separated into two independent fractions by native-PAGE. Both fractions showed histamine release activity for basophils of Patient-2. Moreover, the binding of serum IgE of Patient-2 to 15 kDa, 28 kDa and 54 kDa proteins were inhibited by the pre-treatment with either rPv or r β '-c. These results suggest that 15 kDa, 28 kDa and 54 kDa proteins are truncated ones containing structures of Pv and β '-c, and produced in incomplete digestion of enzymatic processing of the precursor Vg proteins. Actually, a decrease of the molecular weight of the 28 kDa protein by dephosphorylation implies that the 28 kDa protein has a component of Pv, which harbors the most abundantly phosphorylated sites of serines in Vg fragments. The putative molecular weight of the fragment containing amino acid sequence of Pv and β '-c was calculated as more than 25 kDa (Supplementary Fig. 1), while the molecular weight of antigens detected in denatured immunoblotting were 15 kDa, 28 kDa and 54 kDa. The discrepancy between molecular weight of estimation and the 15 kDa antigen is not fully explained but may be affected by the structure of the protein or the treatments of sample preparation.

The comparison of the amino acid sequence showed that the sequences among various fish species of Pv had the lowest homogeneity in Vg fragments between walleye pollock and rainbow trout (salmon) with amino acid substitutions and insertions (Supplementary Fig. 4). Actually, both serum IgE of Patient-1, who showed clinical reactivity to GC roe and salmon roe, and that of Patient-3, who showed reactivity to salmon but not to GC roe, showed the IgE binding to r β '-c but not to rPv. On the other hand, serum IgE of Patient-2 who responded specifically to GC roe showed the IgE binding to both rPv and r β '-c. Taking into account the high homogeneity among fish roes in β '-c, the epitope for the patient's GC roe-specific reactivity is likely in the site of Pv with some reactivity to a part of β '-c. It is feasible that a part of β '-c may be associated with the antigenic ability of Pv in sensitization and reactivity to fish roe.

Although immunological blotting and its inhibition assays by roe extracts and recombinant proteins revealed specific bindings of Patient-2's IgE to Pv and β '-c of GC roe, neither rPv nor r β '-c induced significant histamine release from basophils of Patient-2. This might be due to the addition of TF to the recombinant proteins. TF were overexpressed to prevent aggregation and promote production of recombinant proteins in soluble form in *E. coli*, but the molecular weight of TF is large (58 kDa) and may affect the steric structure of rPv and r β '-c. Moreover, recombinant proteins expressed in *E. coli* are not subjected to post-translational modification. These difference between artificial recombinant proteins and native proteins in GC roe may result in the impaired histamine release by the recombinant ones.

The limitation of this study is that the rarity of patients with hypersensitivity specifically to GC roe did not allow us to analyze sera of a number of patients to solidly conclude cross-reactivity with other fish roes. Nevertheless, we revealed that 15 kDa, 28 kDa and 54 kDa Vg fragments harboring structures of Pv and β '-c and their phosphorylation could comprise antigens for Patient-2 with specific hypersensitivity to GC roe.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2019.09.007>.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

KH, ST, AT, YY, and MH designed the study and wrote the manuscript, KH, KI, YC and MN contributed to data collection. All authors read and approved the final manuscript.

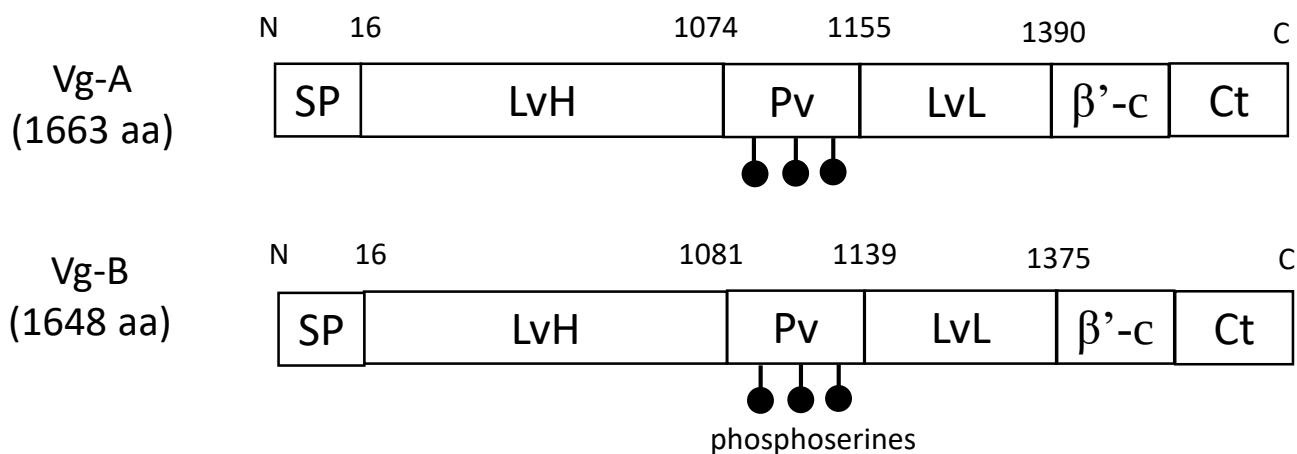
References

- Imai T, Kaneko H. [Commentary on Japanese Pediatric Guideline for Food Allergy 2016 Chapter 3. Epidemiology and natural course]. *Jpn J Pediatr Allergy Clin Immunol* 2017;**31**:188–92 (in Japanese).
- Shimizu Y, Nakamura A, Kishimura H, Hara A, Watanabe K, Saeki H. Major allergen and its IgE cross-reactivity among salmonid fish roe allergy. *J Agric Food Chem* 2009;**57**:2314–9.
- Wallace RA. Vitellogenesis and oocyte growth in nonmammalian vertebrates. *Dev Biol (NY 1985)* 1985;**1**:127–77.
- Kubo T, Watanabe T, Hara A, Shimizu H, Saeki H. [Identification of major allergen in chum salmon roe]. *Bull Fish Sci Hokkaido Univ* 2005;**56**:55–9 (in Japanese).
- Saeki H. [Allergen in fish roe]. *[The Allergy Pract]* 2008;**28**:625–30 (in Japanese).
- Kondo Y, Kakami M, Koyama H, Yasuda T, Nakajima Y, Kawamura M, et al. IgE cross-reactivity between fish roe (Salmon, Herring and pollock) and chicken egg in patients anaphylactic to salmon roe. *Allergol Int* 2005;**54**:317–23.
- Makinen-Kiljunen S, Kiistala R, Varjonen E. Severe reactions from roe without concomitant fish allergy. *Ann Allergy Asthma Immunol* 2003;**91**:413–6.
- Shindo H, Ishii K, Yanase Y, Suzuki H, Hide M. Histamine release-neutralization assay for sera of patients with atopic dermatitis and/or cholinergic urticaria is useful to screen type I hypersensitivity against sweat antigens. *Arch Dermatol Res* 2012;**304**:647–54.
- Hide M, Tanaka T, Yamamura Y, Koro O, Yamamoto S. IgE-mediated hypersensitivity against human sweat antigen in patients with atopic dermatitis. *Acta Derm Venereol* 2002;**82**:335–40.
- Shimizu Y, Kishimura H, Kanno G, Nakamura A, Adachi R, Akiyama H, et al. Molecular and immunological characterization of beta'-component (Onc k 5), a major IgE-binding protein in chum salmon roe. *Int Immunol* 2014;**26**:139–47.
- Hara A. [Immunobiochemical studies on folliculogenesis related proteins in fish]. *Nippon Suisan Gakkaishi* 2013;**79**:300–10 (in Japanese).

Supplementary Methods

Protein identification by nano-flow liquid chromatography-mass spectrometry (nanoLC-MS)

Extracted proteins from *Gadus chalcogrammus* (GC) roe were separated by SDS-PAGE, and were stained with silver staining. Four protein bands in each band of the western blotting (15 , 28 , 40 and 70 kDa) with serum IgE of Patient-2 revealed hypothetical proteins were excised from the gel and then were in-gel digested with trypsin after reduction with dithiothreitol and alkylation with iodoacetamide. The tryptic peptides were trapped with a short ODS column (PepMap 100; 5 x 0.3 mm ID, Thermo Fisher Scientific, 30 μ L/min) and separated with a long ODS column (Capillary Column; 120 x 0.075 mm ID, Nikkyo Technos, Tokyo, Japan, 0.2 μ L/min) using nanoLC (Ultimate 3000 RSLC, Thermo Fisher Scientific). The eluate was continuously introduced into a nanoESI source and analyzed by mass spectrometry (MS) and MS/MS (LTQ Orbitrap XL, Thermo Fisher Scientific). The MS and MS/MS spectra were generated in the positive ion mode using Orbitrap (m/z 300-1500) and Iontrap (data-dependent scan of top five peaks using CID), respectively, with the capillary source voltage at 1.5 kV and the transfer capillary temperature at 200°C. The assignment of the MS/MS data to GC roe proteins was performed using the Xcalibur software (version 1.4, Thermo Fisher Scientific). The MS/MS data were searched using Mascot (Matrix Sciences) against Gadidae proteins in UniProt database using Proteome Discoverer software (version 2.2, Thermo Fisher Scientific). Doubly, triply, and quadruply charged peptide ions were subjected to the database search with precursor and fragment ion mass tolerance of ± 10 ppm and ± 0.8 Da, respectively, with static modification (carbamidomethylation of cysteine) and dynamic modification (oxidation of methionine and deamidation of asparagine and glutamine). The significance threshold on Proteome Discoverer for Mascot search was set at $P < 0.05$ (Target Decoy PSM Validator) and two missed trypsin cleavage was allowed.



Supplementary Fig. 1. Structural characterization of vitellogenin A (Vg-A) and Vg-B in *Gadus chalcogrammus*
 SP, signal peptide; LvH, lipovitellin heavy chain; Pv, phosvitin; LvL, lipovitellin light chain; β'-c, β'-component; Ct, an additional C-terminal peptide.

70 kDa

Uniprot					
Accession	Description	Score	Coverage	# UniquePeptides	MW [kDa]
➡ S6A6K5	Vitellogenin A OS=Gadus chalcogrammus GN=VgA PE=2 SV=1 - [S6A6K5_GADCH]	2458.90	23.09 %	13	182.3
➡ S6A926	Vitellogenin B OS=Gadus chalcogrammus GN=VgB PE=2 SV=1 - [S6A926_GADCH]	1193.21	12.26 %	6	179.1
G8DZS1	Heat shock cognate 70 kDa protein (Fragment) OS=Gadus morhua PE=2 SV=1 - [G8DZS1_GADMO]	891.56	43.88 %	11	30.7
Q92079	Serotransferrin (Fragment) OS=Gadus morhua GN=tf PE=2 SV=1 - [TRFE_GADMO]	816.24	25.55 %	13	67.9

40 kDa

Uniprot					
Accession	Description	Score	Coverage	# UniquePeptides	MW [kDa]
➡ S6A6K5	Vitellogenin A OS=Gadus chalcogrammus GN=VgA PE=2 SV=1 - [S6A6K5_GADCH]	3662.09	35.72 %	29	182.3
➡ S6A926	Vitellogenin B OS=Gadus chalcogrammus GN=VgB PE=2 SV=1 - [S6A926_GADCH]	2588.34	24.51 %	13	179.1
G8DZS1	Heat shock cognate 70 kDa protein (Fragment) OS=Gadus morhua PE=2 SV=1 - [G8DZS1_GADMO]	908.83	46.04 %	11	30.7
Q76LU1	Alpha skeletal actin-1 OS=Gadus chalcogrammus PE=2 SV=1 - [Q76LU1_GADCH]	285.19	22.28 %	7	41.9
Q8AV86	Tropomyosin OS=Gadus chalcogrammus PE=2 SV=1 - [Q8AV86_GADCH]	129.84	16.55 %	3	32.6
P26325	Alcohol dehydrogenase 1 OS=Gadus morhua subsp. callarias PE=1 SV=1 - [ADH1_GADMC]	64.34	3.47 %	1	40.1

28 kDa

Uniprot					
Accession	Description	Score	Coverage	# UniquePeptides	MW [kDa]
➡ S6A926	Vitellogenin B OS=Gadus chalcogrammus GN=VgB PE=2 SV=1 - [S6A926_GADCH]	1031.39	12.74 %	5	179.1
➡ S6A6K5	Vitellogenin A OS=Gadus chalcogrammus GN=VgA PE=2 SV=1 - [S6A6K5_GADCH]	928.77	8.96 %	21	182.3
Q76LU1	Alpha skeletal actin-1 OS=Gadus chalcogrammus PE=2 SV=1 - [Q76LU1_GADCH]	117.11	6.90 %	2	41.9
V9MA55	Proliferating cell nuclear antigen (Fragment) OS=Gadus morhua GN=PCNA PE=2 SV=1 - [V9MA55_GADMO]	70.94	9.85 %	1	14.4
Q98954	Heavy-chain myosin (Fragment) OS=Gadus morhua GN=MHC PE=2 SV=1 - [Q98954_GADMO]	24.72	7.34 %	1	25.1

15 kDa

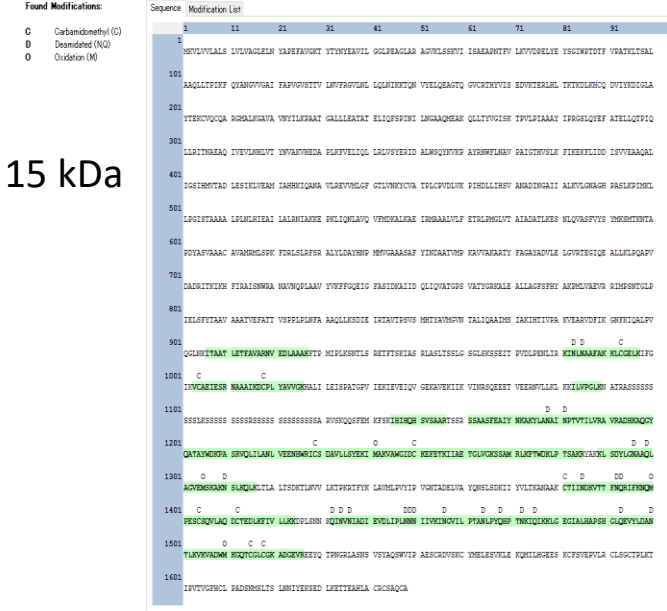
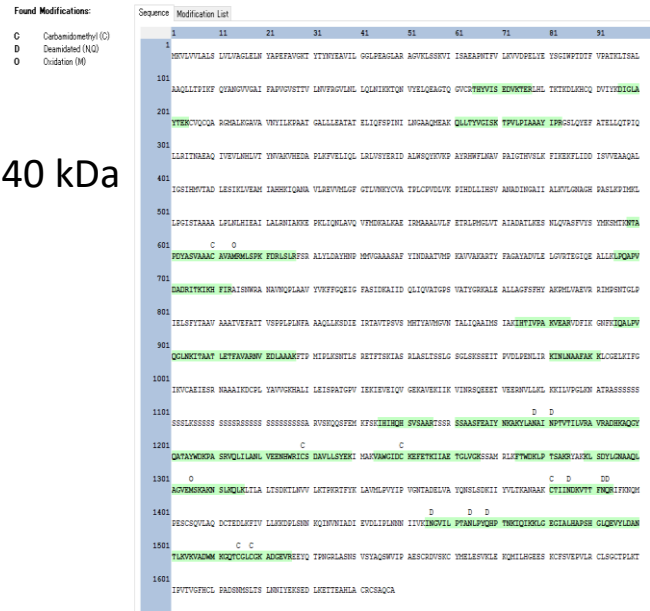
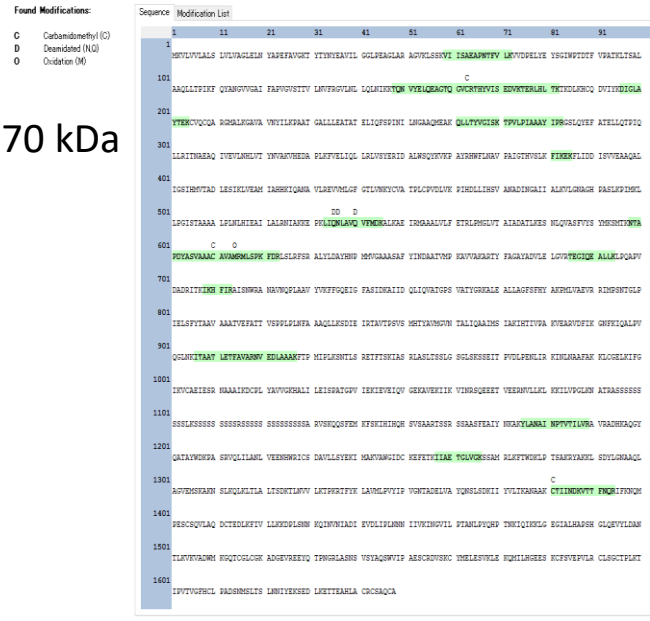
Uniprot					
Accession	Description	Score	Coverage	# UniquePeptides	MW [kDa]
➡ S6A926	Vitellogenin B OS=Gadus chalcogrammus GN=VgB PE=2 SV=1 - [S6A926_GADCH]	4924.19	23.18 %	22	179.1
➡ S6A6K5	Vitellogenin A OS=Gadus chalcogrammus GN=VgA PE=2 SV=1 - [S6A6K5_GADCH]	3245.33	18.28 %	36	182.3
Q76LU1	Alpha skeletal actin-1 OS=Gadus chalcogrammus PE=2 SV=1 - [Q76LU1_GADCH]	181.92	12.73 %	3	41.9
Q9PUG4	Beta-2 tubulin OS=Gadus morhua PE=2 SV=1 - [Q9PUG4_GADMO]	69.44	3.38 %	1	49.6
S6A416	Phosvitinless vitellogenin OS=Gadus chalcogrammus GN=PvIVg PE=2 SV=1 - [S6A416_GADCH]	56.79	1.18 %	2	139.8
G8DZS2	Nucleoside diphosphate kinase (Fragment) OS=Gadus morhua PE=2 SV=1 - [G8DZS2_GADMO]	34.28	8.63 %	1	15.5
A0A067XL60	Cold inducible RNA binding protein (Fragment) OS=Gadus morhua PE=2 SV=1 - [A0A067XL60_GADMO]	33.08	12.22 %	1	9.6
P52865	60S ribosomal protein L22 (Fragment) OS=Gadus morhua GN=rpl22 PE=2 SV=2 - [RL22_GADMO]	26.45	4.80 %	1	14.5
D7R9W8	Interferon induced with helicase C domain 1 (Fragment) OS=Gadus morhua PE=2 SV=1 - [D7R9W8_GADMO]	23.71	3.46 %	1	26.4

Supplementary Fig. 2. Summary of mass spectrometric analysis

Mass spectrometric amino acid sequence analysis of the protein in each band of the western blotting (15, 28, 40 and 70 kDa) with serum IgE of Patient-2 revealed hypothetical proteins in order of the scores calculated by the comparison of detected partial peptide sequences with database. All bands included vitellogenin-A and B of *Gadus chalcogrammus*.

Found Modifications:		Sequence	Modification List
C	Carbamidomethyl (C)	1	1 11 21 31 41 51 61 71 81 91
D	Deamidated (NQ)	2	
O	Oxidation (M)	101	100
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Amino acid sequences of GC Vg-B fragments detected by mass spectrometry



Supplementary Fig. 3(b). Amino acid sequences of GC Vg-B fragments detected by mass spectrometry Partial peptides of GC vitellogenin-B of *Gadus chalcogrammus* detected by mass spectrometric analysis. The high lighted characters indicate the detected peptide sequences.

 Pv
 LvL
 β' -c

[illegible]

