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Molecular characterization and expression analysis of three subclasses of IgT in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

As the teleost specific immunoglobulin, IgT plays important roles in systemic and mucosal immunity. In the current study, in rainbow trout, we have cloned the heavy chain (Ig τ) genes of a secretory form of IgT2 as well as the membrane and secretory forms of a third IgT subclass, termed IgT3. Conserved cysteine and tryptophan residues that are crucial for the folding of the immunoglobulin domain as well as hydrophobic and hydrophilic residues within CART motif were identified in all IgT subclasses. Through analysis of the rainbow trout genome assembly, Ig τ 3 gene was found localized upstream of Ig τ 1 gene, while Ig τ 2 gene situated on another scaffold. At the transcriptional level, Ig τ 1 was mainly expressed in both systemic and mucosal lymphoid tissues, while Ig τ 2 was largely expressed in systemic lymphoid organs. After LPS and poly (I:C) treatment, Ig τ 1 and Ig τ 2 genes exhibited different expression profiles. Interestingly the transcriptional level of Ig τ 3 was negligible, although its protein product could be identified in trout serum. Importantly, a previously reported monoclonal antibody directed against trout IgT1 was able to recognize IgT2 and IgT3. These data demonstrate that there exist three subclasses of IgT in rainbow trout, and that their heavy chain genes display different expression patterns during stimulation. Overall, our data reflect the diversity and complexity of immunoglobulin in trout, thus provide a better understanding of the IgT system in the immune response of teleost fish.

Keywords

IgT; Subclass; Immunoglobulin; Rainbow trout

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1. Introduction

The adaptive immune system is based on diverse antigen receptors such as the variable lymphocyte receptor (VLR), which is generated via the recombination of leucine-rich repeat (LRR) modular units in jawless vertebrates, as well as B and T cell receptors (BCRs and TCRs), which are generated via somatic hypermutation and variable-diversity-joining rearrangement (VDJ rearrangement) in jawed vertebrates (Alder et al., 2005; Deng et al., 2013). As key molecules in the adaptive immune system, immunoglobulins (Igs) play a pivotal role in the elimination of extracellular and intracellular pathogens. Functional Igs are primarily expressed on the surface of B lymphocytes as BCRs or are secreted into body fluids as antibodies (Abs) to agglutinate pathogens (Parra et al., 2013). The typical structure of an Ig molecule consists of two heavy (H) chains and two light (L) chains. IgH chains usually contain one variable (V) domain and two to four constant (C) domains, and IgL chains are composed of one V domain and one C domain. The classification of an Ig isotype is based on the C domains of its H chain (Danilova and Amemiya, 2009; Flajnik and Kasahara, 2010; Schroeder and Cavacini, 2010).

To date, five Ig isotypes have been well characterized in mammals: IgM (H chain: μ), IgD (δ), IgG (γ), IgE (ϵ) and IgA (α) (Schroeder and Cavacini, 2010). Human and mouse also possess certain subclasses of IgG (French and Harrison, 1984) and IgA (Kett et al., 1986), which enable more subtle immune responses. Among those Igs, IgM and IgD are evolutionarily conserved and are thought to be the primordial Ig isotypes (Edholm et al., 2011; Han et al., 2016). However in teleosts, IgM (Bradshaw et al., 1971; Hordvik et al., 2013), IgD (Edholm et al., 2010; Ramirez-Gomez et al., 2012; Wilson et al., 1997) and IgT or IgZ (H chain: τ or ζ) (Hansen et al., 2005; Hu et al., 2010) have been identified in various fish species. The teleost specific IgT has first been reported in zebrafish (*Danio rerio*) (Danilova et al., 2005) and rainbow trout (*Oncorhynchus mykiss*) (Hansen et al., 2005). After that, IgT has been cloned in various teleost species except for catfish and medaka (Fillatreau et al., 2013). IgT subclasses are also reported in some teleosts such as Atlantic salmon (*Salmo salar*) (Yasuike et al., 2010), stickleback (*Gasterosteus aculeatus*) (Bao et al., 2010; Gambon-Deza et al., 2010) and carp (*Cyprinus carpio*) (including a chimeric IgM-IgT) (Ryo et al., 2010). Thus, Igs in teleost fish may be more complex than previously expected (Sunyer, 2013). IgT plays important roles in mucosal immunity in the gut (Zhang et al., 2010), skin (Xu et al., 2013) and gill (Xu et al., 2016) in rainbow trout (*Oncorhynchus mykiss*). In addition, recent studies indicated that IgT⁺ B cells also participate in the responses against viral infections (Castro et al., 2013) and DNA vaccination (Castro et al., 2014). These evidences indicate that teleost IgT is also involved in systemic immunity (Castro and Tafalla, 2015). In trout, IgT was found in serum as a monomer while in mucus it was characterized as a polymer where the monomers were associated via noncovalent interactions (Zhang et al., 2011).

Two types of IgH locus organization have been reported to date: translocon and cluster (Hikima et al., 2011). IgH genes in tetrapods and teleosts are organized as translocon, with multiple variable (V), diversity (D) and joining (J) segments arrayed upstream of constant (C) gene segments in the following order: (V_n-D_n-J_n-C_n) (Danilova et al., 2005; Mashoof et al., 2014; Sun et al., 2013). The IgH loci of cartilaginous fish exhibit a cluster organization

as follows: (V-D-J-C)_n (Das et al., 2012; Flajnik, 2002). The V, D and J segments are assembled by RAG-1 and RAG-2 through the recognition of recombination signal sequences (RSSs) at the borders of the V, D and J segments (Hikima et al., 2011). In teleosts, Ig τ exons are either located in the 5' regions of the D and J gene segments of Igu and Ig δ in zebrafish (Danilova et al., 2005) and stickleback (Gambon-Deza et al., 2010) or inserted within the V segments of Igu and Ig δ in rainbow trout (Hansen et al., 2005) and Atlantic salmon (Yasuike et al., 2010), which has led to the evolution of a distinct B cell lineage in addition to IgM (Hansen et al., 2005). Furthermore, the heavy chains of teleost IgT subclasses are encoded by duplicated Ig τ genes in the same IgH locus or encoded by different IgH loci (Zhang et al., 2011), potentially resulting from teleost-specific and/or salmonid-specific whole-genome duplication events (Berthelot et al., 2014).

So far in rainbow trout, two IgT subclasses have been reported, the membrane and secretory forms of IgT1 (Hansen et al., 2005) and the membrane form of IgT2 (Danilova et al., 2005). In this study, a third Ig τ gene, encoding the H chain of IgT3, was identified in rainbow trout in addition to the secretory form of IgT2. The existence of three IgT subclasses was verified also at the protein level. The Ig τ gene loci were also characterized based on the trout genome database. All three Ig τ genes could be transcribed and translated into both secretory and membrane forms. Furthermore, to investigate potential functional differences in these IgT subclasses, as well as IgM and IgD, the phylogenetic relationships and expression patterns of trout IgH genes were analyzed.

2. Materials and methods

2.1. Experimental fish and tissue preparation

Rainbow trout (70–100 g) were obtained from Zhanghe Reservoir Rainbow Trout Farm (Jingmen, China) and maintained in aquarium tanks using a water recirculation system involving extensive filtration and temperature control (14–16 °C). The fish were acclimated to the aquarium tanks for 2 weeks before being used in experiments.

Fifty trout were divided into two groups, and intraperitoneally (i.p.) injected with LPS (from *Escherichia coli* 0111:B4; Sigma-Aldrich) and poly (I:C) (a synthetic analogue of viral dsRNA; Sigma-Aldrich), separately, at a dose of 100 μ g per 50 g fish body weight. At 0, 1, 3, 7, 14 and 21 d after injection, three fish from each group were euthanized with tricaine (MS-222, Sigma-Aldrich), and the spleen and gut were collected from euthanized fish under sterile environment. These samples were flash frozen in liquid nitrogen and then transferred to –80 °C freezer for RNA extraction. All animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Chinese Academy of Sciences.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was extracted using the Trizol reagent (Invitrogen) and digested with an RNase-free DNase (Qiagen) according to the manufacturer's instruction. In order to normalize the gene expression level for each sample, same amounts (500 ng) of the total RNA were used for cDNA synthesis in a 25 μ l reaction volume with M-MLV reverse transcriptase

(Promega). The synthesized cDNA was diluted with 75 μ l of RNase-free water and used as template for quantitative real-time PCR (qPCR).

2.3. Cloning of trout Ig τ subclasses

The constant regions of trout Ig τ 1 (GenBank accession No. AY870265, membrane form; AY870263, secretory form) and Ig τ 2 (GenBank accession No. AY773715, membrane form) were used as queries to search trout expressed sequence tag (EST) database at NCBI (<http://www.ncbi.nlm.nih.gov>). To obtain the C region cDNAs of the secretory form of Ig τ 2 and both of the secretory and membrane forms of the currently discovered Ig τ subclass (Ig τ 3), firstly 3' RACE was carried out with the specific forward primers, designed based on the reported Ig τ 2 and the identified Ig τ 3 EST (GenBank accession No. CX150523) sequences, as well as the adaptor primers (Supplemental Table 1). Then the cDNAs encoding a partial VH region and the whole CH region of each Ig τ subclass were amplified with the degenerate forward and specific reverse primers (Supplemental Table 1). Ex Taq HS (Takara) was used in all PCR and the PCR products were cloned into the pMD19-T vector (Takara) for sequencing.

2.4. Sequence and phylogenetic analyses

Sequenced trout Ig τ subclasses were analyzed using DNAMAN software (Lynnon). To translate the nucleotide sequences to amino acid sequences, the ExPASy online tools (<http://web.expasy.org/>) were used. Signal peptides were predicted using the SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>). Multiple sequence alignment was performed using CLUSTAL X version 2.1 with default parameters and then manually adjusted. On the basis of the alignment, phylogenetic tree was constructed with the MEGA 4.0 package, using a neighbor-joining algorithm and pairwise deletion. Bootstrap values supporting tree nodes were derived from 1000 replications. Trout β ₂-microglobulin was used as outgroup.

2.5. Extension and annotation of trout IgH locus

Using the published rainbow trout genome database (Berthelot et al., 2014), we extended the trout IgH locus (Hansen et al., 2005) by assembling scaffold_2931 together with the previously reported trout IgH locus (GenBank accession No. AH014877). The software GENSCAN and pairwise TBLASTN alignments were employed for the assembled sequence annotation as described previously (Zhang et al., 2016). According to conserved recombination signal sequence (RSS) motif specificity, D and J segments were identified by pattern search with the program FUZZNUC, an online software package (<http://bioinfo.nhri.org.tw/cgi-bin/emboss/fuzznuc>). The pattern of RSS heptamer and nonamer sequences with an 11- to 13-nucleotide spacer (CACAGTG-N₁₁₋₁₃-ACAAAACC) allowing a maximum of four mismatches and then manually adjusted.

2.6. qPCR analysis of IgH expression

In order to determine the expression levels of trout IgH genes in different tissues and sorted cells, qPCR was performed with CFX Connect™ (BioRad). The efficiencies for all qPCR primers were analyzed as previously described (Zhang et al., 2009). The primer pairs for IgM, IgD, β -actin and EF-1 α were reported previously (Chang et al., 2013; Zhang et al.,

2010). All the primer pairs used in this study were qualified, with the efficiencies between 90% and 105%. The specificity of each primer pair was verified by dissociation curve and sequence analyses of the qPCR products. The qPCRs were conducted as follow: 4 μ l diluted cDNA template, 5 μ l SsoAdvanced™ SYBR Green Supermix (BioRad), and 250 nM forward and reverse primers in 10 μ l reaction volumes. The amplification reaction condition consisted of 95 °C for 3 min, 45 cycles of 95 °C for 20 s and 60 °C for 20 s, and then 72 °C for 20 s. Melt curve from 65 °C to 95 °C was added in the final. Three independent qPCR experiments were performed.

2.7. Leukocyte isolation and fractionation

Trout leukocytes were isolated from the peripheral blood (PBLs) and head kidney (HKLs) as described previously (Boshra et al., 2004, 2005). Briefly, trout blood was obtained from the caudal vein and diluted (1/5) immediately with DMEM (Invitrogen) supplemented with 100 μ g/ml streptomycin, 100 U/ml penicillin, and 25 U/ml heparin (Invitrogen). Trout head kidney was removed aseptically and passed slowly through a 100 mm nylon mesh (BD Biosciences), then suspended in the same culture medium as described above. The diluted cell suspensions from blood and head kidney were carefully layered onto a 51/34% discontinuous Percoll (GE Healthcare) density gradient and then centrifuged, with zero accelerate and brake, at 400 *g* for 30 min. The cells laying at the interfaces were carefully collected, and immediately washed with DMEM supplemented with 100 μ g/ml streptomycin and 100 U/ml penicillin.

Cell sorting was carried out using the conditions described previously (Zhang et al., 2010). Briefly, isolated trout PBLs and HKLs were suspended in sterility PBS (Invitrogen) supplemented with 2% fetal bovine serum (FBS; Invitrogen), and then stained with mouse anti-trout IgT (Clone: 38.5; isotype: IgG2b) (1 μ g/ml) monoclonal antibody (mAb) at 4 °C for 60 min. As a control, cells were incubated with normal mouse IgG. FITC-goat anti-mouse IgG2b (Jackson Scientific) was used as secondary antibody to detect the IgT⁺ cells. After two times of washing with PBS supplemented with 2% FBS, the IgT⁺ cells from trout PBLs and HKLs were sorted by flow cytometry using BD FACSAria III (BD Biosciences). The sorted cells were washed and analyzed by qPCR.

2.8. Prokaryotic expression of the recombinant heavy chains of trout IgT subclasses

The subcloning and expression of the heavy chains of trout IgT subclasses in *Escherichia coli* were performed as described previously (Zhang et al., 2009) with minor modification. The pET-23b(+) expression vector (Novagen) was used for constructing the expression plasmids. The DNA fragments encoding the C τ 2~C τ 4 of trout IgT1, IgT2 and IgT3 were amplified and digested with *EcoR*I and *Xho*I and ligated to pET-23b(+) vector, which was digested with the same restricted enzymes. Then the recombinant plasmids were verified by sequencing. The expression plasmids were subsequently transformed in BL21(DE3)-CodonPlus-RIL expression host competent cells (Stratagene). Bacterial cells were cultured in Luria-Bertani broth containing 100 μ g/ml ampicillin and 34 μ g/ml chloramphenicol and grown at 37 °C with shaking. The OD₆₀₀ of the culture was allowed to reach 0.6 before induction with 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and allowed to grow for additional 15 h at 15 °C. Cells were harvested by centrifugation and resuspended in PBS

(containing the protease inhibitor mixture, Sigma-Aldrich). Cell suspensions were disrupted using sonication and then centrifuged at 12000 *g* for 20 min to pellet the precipitate. Finally, the supernatants of cell lysates were quantified and subjected to SDS-PAGE for coomassie blue staining and immunoblot analysis.

2.9. Immunoblot analysis

The supernatants (containing the soluble recombinant proteins) of cell lysates were separated by 12% SDS-PAGE under reducing and non-reducing conditions and then transferred to polyvinylidene difluoride membranes (BioRad). The membranes were blocked in TBST buffer (25 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20 [pH 7.5]) containing 5% nonfat dry milk overnight at 4 °C, and then probed with diluted mouse anti-trout IgT mAb (Clone: 38.5; isotype: IgG2b) for 2 h at room temperature. The membranes were washed three times with TBST and incubated with peroxidase-conjugated anti-mouse IgG (NA931; GE Healthcare) for 1 h at room temperature. After three additional washes with TBST, the membranes were stained with Immobilon TM Western Chemiluminescent HRP Substrate (Millipore) and detected using an ImageQuant LAS 4000 system (GE Healthcare).

2.10. Serum IgT purification and LC-MS/MS

Purification and LC-MS/MS analysis of trout serum IgT were performed as described previously (Zhang et al., 2010). Approximately 2 µg of affinity purified trout IgT was resolved on a 4–15% SDS-PAGE under non-reducing conditions and stained with Coomassie blue. The band corresponding to IgT was subjected to in-gel digestion and LC-MS/MS analysis, respectively. The resulting masses and MS/MS spectra were searched against the trout Ig τ subclass sequences.

2.11. Statistical analysis

The statistic *p* values were calculated by Student's *t*-test (SPSS Statistics, version 19, IBM) between different groups. The *p* value < 0.05 was considered statistically significant.

3. Results

3.1. Sequence analysis of trout Ig τ subclasses

Based on the reported cDNA sequences of trout Ig τ 1 (membrane and secretory forms) and Ig τ 2 (membrane form), the cDNAs encoding a partial VH region and the whole CH region of the secretory form of Ig τ 2 and both of the secretory and membrane forms of the currently discovered Ig τ 3 were cloned.

To characterize the H chains of trout IgT subclasses, alignment and similarity analyses of the deduced amino acid sequences of the CH domains, secretory tail and transmembrane (TM) region were performed between trout IgM and IgT as well as zebrafish IgM and IgZ. As shown in Fig. 1, trout IgT3 is composed of four putative CH domains, same as IgT1 and IgT2. Within CH1 domain, the first cysteine residue that is considered to be used to form interchain disulfide bond is conserved in all aligned Ig sequences. In addition, conserved cysteine residues (crucial for the forming of intrachain disulfide bonds) and tryptophan residues (crucial for the folding of IgSF domain) (Lesk and Chothia, 1982) are also present

within each CH domain of trout IgT3. Furthermore, the TM region of trout IgT3 contains a conserved antigen receptor transmembrane (CART) motif, which is present in the TM regions of all antigen receptors of jawed vertebrates.

A similarity comparison of the CH domains within the IgM and IgT subclasses in trout and zebrafish was carried out (Fig. 2). The deduced amino acid sequences of CH1 share the highest similarity among trout IgT subclasses, with IgT1 even reaching 90% of identity with IgT3. However, the CH4 domains, whose identities are less than 80%, exhibited the lowest similarity among trout IgT subclasses. The similarities of the CH2 and CH3 domains are intermediate to those of CH1 and CH4. When the whole CH regions of IgT subclasses in trout are compared, the sequence identity between IgT1 and IgT3 is slightly higher than the identities between IgT2 and IgT3 as well as between IgT2 and IgT1.

To explore the relationships between trout Ig τ subclasses and other teleost IgH, a phylogenetic analysis was carried out based on the CH regions of diverse teleost species (Fig. 3). The resulting phylogenetic tree indicated that trout Ig τ subclasses are closely related to salmon Ig τ subclasses and cluster with the Ig τ clade of other teleosts. The currently cloned trout Ig τ 3 is more closely related to trout Ig τ 1 than to Ig τ 2.

3.2. Genomic organization of trout IgH locus

To identify the Ig τ 2 and Ig τ 3 gene loci in trout genome, local blast searching was performed against rainbow trout genome sequences and scaffold database. According to the blast results, the CH regions of the secretory and membrane forms of trout IgT3 are encoded by the same C τ 3 gene through alternative splicing at the 3' end of the CH4 exon. The secretory Ig τ 3 transcripts are produced through the strategy that the splice site at the end of CH4 exon is ignored and the transcription continues until reaching the stop codon, whereas the transcripts of the membrane form of the Ig τ 3 are produced via splicing of the CH4 exon to the TM exon.

In addition, exons of both C τ 1 and C τ 3 were identified in the same scaffold_2931 (GenBank accession No. FR907203), whereas the Ig τ 2 locus is located in trout scaffold_3178 (GenBank accession No. FR907450) (Fig. 4). Thus, the Ig τ locus within trout scaffold_2931 can be assembled with the previously reported trout IgH locus (AH014877). Based on the extended trout IgH locus, the Ig τ 3 gene is located upstream of the Ig τ 1 gene and possesses its own V, D and J gene segments, while the relative location of Ig τ 2 gene with Ig τ 1 and Ig τ 3 is still unclear.

3.3. Tissue distribution of trout IgH

The expression patterns of the IgH genes were explored using qPCR. As shown in Fig. 5, trout IgH genes were primarily expressed in lymphoid tissues, such as the head kidney, spleen, thymus, gill and blood. Specifically, Ig μ transcripts were largely detected in the head kidney, spleen and gill, and moderately in the thymus, blood and gut. Ig δ was expressed at considerably high levels in the head kidney, spleen and blood. However, Ig τ 1 and Ig τ 2 demonstrated different expression patterns. Ig τ 1 transcripts were found not only in the systemic lymphoid tissues (such as head kidney, spleen, thymus and blood) but also in the mucosal lymphoid tissues (such as gut, gill and skin). However, Ig τ 2 transcripts were

primarily detected in the systemic lymphoid tissues such as spleen, thymus and blood. In contrast, Ig τ 3 expression levels were too low to be detected except for a slight transcription in the spleen (data not shown).

3.4. Regulation of trout IgH gene expression by LPS and poly (I:C)

Expression levels of the IgH genes in trout gut and spleen were examined after i.p. injection of LPS or poly (I:C) at different time points (Fig. 6). Trout Ig μ transcripts in the gut were substantially increased at 1 d after stimulation with LPS and reached a peak at 3 d, whereas its expression in the spleen was obviously increased at 7 d. When stimulated with poly (I:C), trout Ig μ was also up-regulated in the gut at 3 d, but almost no change was observed in the spleen. In the LPS treatment group, the expression of Ig δ in the gut and spleen exhibited a similar pattern and slowly increased to a peak at 7 d and 14 d, followed by a decrease at 21 d. After stimulation with poly (I:C), the expression levels of Ig δ fluctuated regularly in the spleen but obviously increased in the gut at 14 d. Ig τ 1 transcripts reached a peak in the gut at 3 d after stimulation with LPS and exhibited a slight increase in the spleen, whereas Ig τ 1 was slowly up-regulated and reached a peak at 7 d in the spleen and at 14 d in the gut after treatment with poly (I:C). Furthermore, after LPS stimulation, Ig τ 2 exhibited an obvious increase at 3 d in the gut. However, when stimulated with poly (I:C), Ig τ 2 increased slightly and reached a peak at 7 d in both the spleen and gut. Taken together, trout IgH responses were much stronger in the gut than in the spleen following i.p. injection of LPS and poly (I:C). In addition, Ig τ 1 and Ig τ 2 exhibited differential response patterns.

3.5. Reactivity of the anti-trout IgT1 mAb to IgT2 and IgT3

A mAb directed to trout IgT1 was developed in our previous study (Zhang et al., 2010). With the discovery and classification of three trout IgT subclasses, we were eager to determine the reactivity of this anti-IgT1 mAb with IgT2 and IgT3. First, IgT⁺ B cells were sorted from trout PBLs and HKLs using the mouse anti-trout IgT1 mAb. Thereafter, the expression level of each Ig τ subclass in the sorted IgT⁺ B cells was determined using qPCR. As shown in Fig. 7, Ig τ 1 and Ig τ 2 transcripts were both detected in the IgT⁺ B cells from both the HKLs and PBLs. Ig τ 1 expression levels were higher than that of Ig τ 2 in HKLs, whereas both Ig τ 1 and Ig τ 2 demonstrated equivalent transcriptional levels in PBLs. Ig τ 3 transcripts were too low to be detected using qPCR.

To further investigate the reactivity of the anti-trout IgT1 mAb against the three IgT subclasses, the CH2–CH4 domains of IgT1, IgT2 and IgT3 were subcloned into a prokaryotic expression plasmid, and the soluble expression products were analyzed by Western blotting. As shown in Fig. 8, the recombinant proteins were expressed in soluble form and had a molecular mass of ~40 kDa, and Western blotting revealed an obvious band in the target area corresponding to Ig τ 1, Ig τ 2 and Ig τ 3 recombinant proteins. Therefore, the mAb that we developed to target IgT1 also recognized the recombinant Ig τ 2 and Ig τ 3.

3.6. Identification of trout IgT subclasses at the protein level

Three IgT subclasses were identified at the protein level in trout serum via LC-MS/MS using affinity purified trout serum IgT by the anti-IgT1 mAb. Mass spectrometry analysis identified a total of 10, 1, and 7 unique peptides belonging to the CH domains of Ig τ 1, Ig τ 2

and Ig τ 3, respectively (Table 1), Additionally, 2 peptides were identified to be shared by both Ig τ 1 and Ig τ 3, 1 peptide shared by both Ig τ 2 and Ig τ 3, and 2 peptides shared by all three Ig τ subclasses. These results supported the existence of three IgT subclasses in rainbow trout and provided complementary evidence that the reactivity of the anti-trout IgT1 mAb with trout IgT2 and IgT3.

4. Discussion

IgT is the most recent immunoglobulin class to be identified in a vertebrate species (Flajnik, 2005), and its genomic organization, protein structure and biochemical functions have been characterized in various teleosts. The mucosal immune function of IgT has been well-characterized in trout gut (Zhang et al., 2010), skin (Xu et al., 2013) and gill (Xu et al., 2016), whereas IgM plays a prevalent role in the systemic immunity. More than one subclass of IgT has been identified in different teleosts, which may be attributable to whole genome duplications. The H chains of IgT subclasses are encoded by duplicated Ig τ genes either in the same IgH locus, as in stickleback fish (Bao et al., 2010; Gambon-Deza et al., 2010), or in different IgH loci, as in Atlantic salmon (Yasuike et al., 2010). In the current study, we cloned the H chain genes of the secretory IgT2 as well as the secretory and membrane forms of IgT3 in trout. The H chains of both the secretory and membrane forms of each IgT subclass are encoded by the same Ig τ gene through differential splicing site. The transcript of the H chain of the membrane form of each IgT subclass is produced by splicing the C τ 4 exon to the TM exon, similar to mammalian membrane IgM but different from teleost membrane IgM; however, the generation of secretory IgT involves the same mechanism as teleost secretory IgM. Based on the alignment of the CH domains of IgM and IgT subclasses, conserved cysteine residues that participate in the formation of intrachain disulfide bonds were found within the CH1, CH2 and CH4 domains of both IgM and IgT. However, in the CH3 domains of zebrafish IgZ1 and IgZ2, the positions of the first cysteine residues are different with other sequences, this situation may indicate that non-classical cysteine residues are not important for the formation of the three-dimensional structure of IgSF (Danilova and Amemiya, 2009). A conserved cysteine residue responsible for the interchain disulfide bond with IgL was also found in the CH1 domains of all IgT subclasses. The CART motifs identified in the TM regions of trout IgT subclasses are quite similar to those found in mammalian Igu chains. Thus, the membrane forms of trout IgT subclasses may have the capacity to interact with Ig α /Ig β to form BCRs (Campbell et al., 1994).

Alignment of the CH sequences of IgM and IgT in trout and zebrafish indicates that the CH1 is the most highly conserved domain between different IgH isotypes. However, the CH4 domain exhibits the lowest level of sequence identity, not only between different IgT subclasses but also between IgM and IgT, implying that C τ 4 provides the specificity to IgT and its subclasses (Gambon-Deza et al., 2010). The sequence identities of the whole CH regions of IgM and IgT indicate that among the three IgT subclasses in trout, the relationship between IgT1 and IgT3 is much closer than the relationships between either of them to IgT2. In addition, IgT1 and IgT3 are grouped together in the phylogenetic tree, supporting the sequence identity results.

Knowledge of trout IgH loci was extended with the discovery of the Ig τ 3 gene using local blast. The Ig τ 3 gene contains its own V, D and J segments and is located upstream of Ig τ 1 gene. The Ig τ 2 gene identified in another scaffold indicates that at least two IgH loci may exist in the trout genome (Hansen et al., 2005). This phenomenon was also observed in Atlantic salmon, whose two IgH loci contain 3 functional Ig τ genes and additional 5 Ig τ pseudogenes (Yasuike et al., 2010). The presence of multiple Ig τ genes in trout and salmon might be attributable to teleost-specific and salmonid-specific whole genome duplication events (Berthelot et al., 2014; Hoegg et al., 2004), resulting in a positive and significant increase in the diversity of antibody repertoire. Given the limited information available in terms of trout genome data, further investigation is required to determine if there is any other Ig gene located upstream and downstream of the Ig τ 2 gene.

The heavy chain genes of zebrafish IgZ1 and IgZ2 were reported to exhibit different expression patterns. IgZ1 was predominantly expressed in the head kidney and thymus, whereas IgZ2 was expressed in both the systemic and mucosal lymphoid organs, similar to IgM (Hu et al., 2010). In the current study, the heavy chain genes of trout IgT1 and IgM demonstrated a similar expression pattern with zebrafish IgZ2 and IgM. Trout IgT2 was primarily expressed in the systemic lymphoid organs, like zebrafish IgZ1. This phenomenon may suggest the functional difference between these two IgT subclasses as well as the non-mucosal role for IgT (Castro et al., 2013, 2014; Castro and Tafalla, 2015). In addition, combined with the previous studies about trout IgT⁺ B cells in various mucosal tissues during homeostatic conditions (Xu et al., 2013, 2016; Zhang et al., 2011), it seems that there are more IgT⁺ B cells and IgT transcripts in gill than gut and skin. This may be due to the circulation of B cells in gill (Gomez et al., 2013). Furthermore, IgT3 is slightly expressed only in the spleen. However, the identification of IgT3 peptides in trout serum indicates that its function can not be ignored. Beyond this, transcripts of IgM and IgT could also be detected in trout thymus, where T cells mainly develop. This was also observed in other teleost, like zebrafish (Danilova et al., 2005) and Atlantic salmon (Tadiso et al., 2011). IgM⁺ B lymphocytes could also be detected in the thymus of zebrafish (Danilova and Steiner, 2002), trout (Korytar et al., 2013) and New Zealand groper (*Ployprion oxygeneios*) (Parker et al., 2012). Thus, whether IgT⁺ B cells exist in fish thymus needs further investigation.

When stimulated with *E. coli* LPS and poly (I:C), the expression patterns of trout IgH genes were notably different, suggesting that all IgH genes are involved in the immune responses in rainbow trout. After stimulated with LPS, the expression levels of IgT1 and IgT2 in trout gut were obviously up-regulated, but the regulation of poly (I:C) to IgT1 and IgT2 was weaker. Compared with the previously reported immune responses to parasites in gut (Zhang et al., 2011), skin (Xu et al., 2013) and gill (Xu et al., 2016), it seems that IgT1 and IgT2 play active roles in the immune response of trout to bacteria and parasites in mucosal tissues. In addition, the responses of trout IgT subclasses to LPS stimulation were similar to zebrafish IgZ1 and IgZ2, which could also be obviously stimulated by LPS with different patterns (Hu et al., 2010). This might be due to that LPS is a typical polyclonal activator which could induce the activation of B cells in gut and skin (Hu et al., 2010; Moller, 1965).

In our previous studies we developed a mAb against trout IgT1, and a hybridoma clone that produced a specific antibody with a high affinity for IgT1 was selected (Zhang et al., 2010).

In the current study, IgT⁺ B cells stained with the anti-IgT1 mAb were sorted from trout HKLs and PBLs and found to express Ig τ 1 and Ig τ 2. More interestingly, the anti-IgT1 mAb could recognize all the three recombinant Ig τ and trout serum IgT purified with the anti-IgT1 mAb also contained all the three Ig τ . Thus, the reactivity of anti-IgT1 mAb with all three IgT subclasses is potentially attributed to the same epitope shared by all of the three Ig τ subclasses, which may locate at the CH3 domains.

In summary, a total of three IgT subclasses were characterized in rainbow trout at the nucleotide and protein levels, thus expanding our knowledge of the trout IgH loci. In addition, expression analyses of trout IgH genes in immune tissues under normal and stimulated conditions indicated differential participation of the IgH genes during immune responses. However, further questions remain to be addressed to understand the regulation of expression and functional differences between various IgT subclasses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The nucleotide sequences of the secretory form of Ig τ 2 and the membrane and secretory forms of Ig τ 3 in trout have been submitted to GenBank with accession numbers KX185935 to KX185937, respectively.

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Abbreviations

BCR	B cell receptor
C domain	constant domain
CH	heavy chain constant domain
CL	light chain constant domain
D segment	diversity segment
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
J segment	joining segment
LC	liquid chromatography
LLR	leucine-rich repeat
mAb	monoclonal antibody
MS	mass spectrometry
qPCR	quantitative real-time PCR
RACE	rapid-amplification of cDNA end
RAG	recombination-activating gene
RSS	recombination signal sequence
V domain	variable domain
VH	heavy chain variable domain
VL	light chain variable domain
VLR	variable lymphocyte receptor

V segment variable segment

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A

CH1

	Trout-IgT1	Trout-IgT2	Trout-IgT3	Zfish-IgM	Zfish-IgZ1	Zfish-IgZ2
Trout-IgM	51.0	52.0	52.0	43.5	22.4	33.6
Trout-IgT1		83.6	90.8	41.5	20.2	31.3
Trout-IgT2			83.6	39.6	21.2	33.3
Trout-IgT3				38.6	21.2	30.3
Zfish-IgM					27.7	30.6
Zfish-IgZ1						39.1

CH2

	Trout-IgT1	Trout-IgT2	Trout-IgT3	Zfish-IgM	Zfish-IgZ1	Zfish-IgZ2
Trout-IgM	17.6	21.0	16.8	26.8	13.4	14.2
Trout-IgT1		77.5	81.6	22.6	32.3	27.2
Trout-IgT2			84.6	21.7	34.3	29.2
Trout-IgT3				24.3	30.3	30.3
Zfish-IgM					13.9	17.3
Zfish-IgZ1						46.3

CH3

	Trout-IgT1	Trout-IgT2	Trout-IgT3	Zfish-IgM	Zfish-IgZ1	Zfish-IgZ2
Trout-IgM	12.7	14.1	12.7	28.0	18.2	14.5
Trout-IgT1		76.9	83.6	16.6	23.3	21.4
Trout-IgT2			78.8	20.1	22.1	22.4
Trout-IgT3				17.5	22.4	21.4
Zfish-IgM					13.7	11.1
Zfish-IgZ1						47.6

CH4

	Trout-IgT1	Trout-IgT2	Trout-IgT3	Zfish-IgM	Zfish-IgZ1	Zfish-IgZ2
Trout-IgM	22.8	22.8	21.9	39.4	20.1	21.0
Trout-IgT1		76.4	74.5	16.6	32.0	34.9
Trout-IgT2			75.4	21.2	30.0	33.9
Trout-IgT3				22.2	34.9	39.8
Zfish-IgM					18.6	18.6
Zfish-IgZ1						78.7

B

	Trout-IgT1	Trout-IgT2	Trout-IgT3	Zfish-IgM	Zfish-IgZ1	Zfish-IgZ2
Trout-IgM	24.6	26.1	24.1	36.2	16.4	18.9
Trout-IgT1		78.6	81.8	21.7	26.8	29.0
Trout-IgT2			80.0	23.8	26.7	30.0
Trout-IgT3				23.1	26.6	29.8
Zfish-IgM					17.1	18.5
Zfish-IgZ1						52.7

Fig. 2.

Percentage identity of the amino acid sequences of the CH domains of IgM and IgT/Z in zebrafish and trout. Multiple sequence alignments were performed using CLUSTAL X version 2.1 with default parameters, then the percentage identity (%) was calculated by BioEdit software at default settings (<http://www.mbio.ncsu.edu/BioEdit/BioEdit.html>). The identity that greater than 80% is shaded. (A) The percentage identity within each CH domain; (B) The percentage identity of the whole CH region.

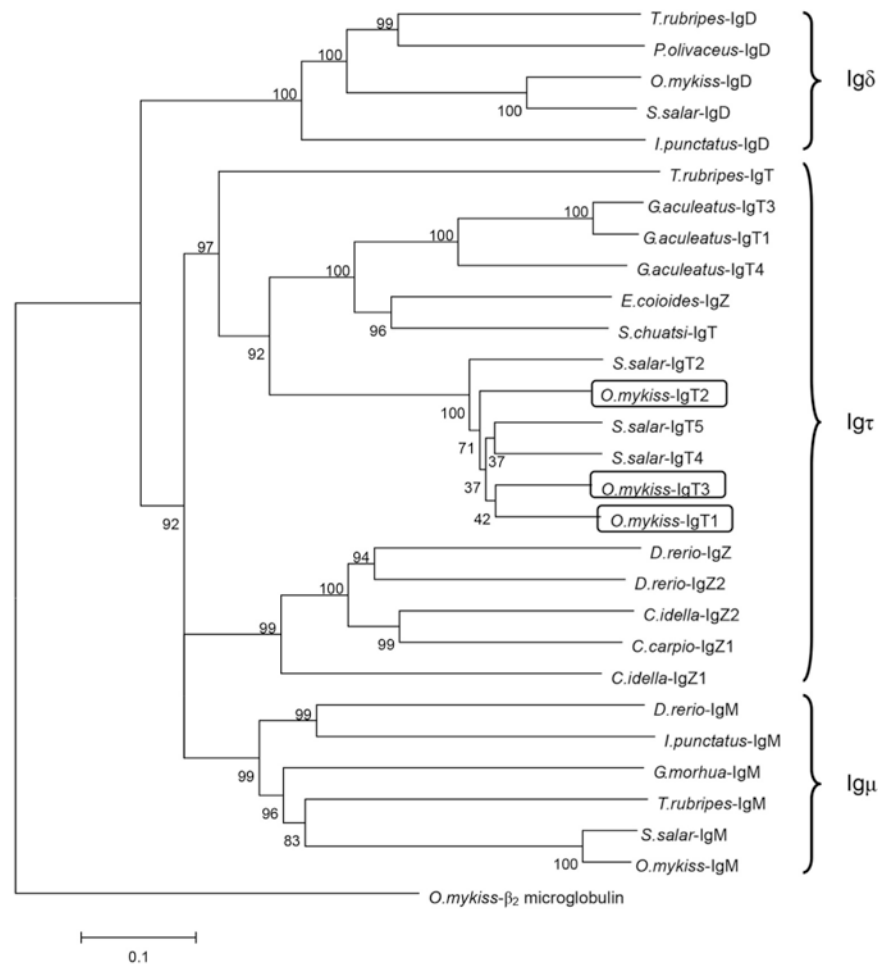


Fig. 3. Phylogenetic analysis of teleost IgH based on the CH sequences. Neighbor-joining tree with pairwise gap deletions was drawn using MEGA 4.0 with 1000 bootstrap replications. Trout β_2 -microglobulin was used as an outgroup. The scale represents the genetic distance. Clusters of sequences are marked and the GenBank accession numbers are: *O. mykiss* β_2 -microglobulin (AAB04661); *T. rubripes* IgD (BAD34542.1); *P. olivaceus* IgD (BAB41204.1); *O. mykiss* IgD (AAW66976.1); *S. salar* IgD (ADD59896.1); *I. punctatus* IgD (ADF56020.1); *D. rerio* IgM (AAT67447.1); *I. punctatus* IgM (A45804); *G. morhua* IgM (AKL81191.1); *T. rubripes* IgM (BAD26619.1); *S. salar* IgM (ACN10415.1); *O. mykiss* IgM (AAW66972.1); *T. rubripes* IgT (BAD69712); *E. coioides* IgZ (ACZ54909); *S. chuatsi* IgT (AAY42141); *S. salar* IgT2 (ADD59873); *S. salar* IgT4 (ADD59858); *S. salar* IgT5 (ADD59859); *D. rerio* IgZ (AAT67446); *D. rerio* IgZ2 (ACH92959); *C. idella* IgZ (ADD82655); *C. idella* IgZ2 (ABF19723); *C. carpio* IgZ1 (BAJ41037); *O. mykiss* IgT1 (AAW66978); *O. mykiss* IgT2 (AAV48553); *O. mykiss* IgT3 (ANW11927).

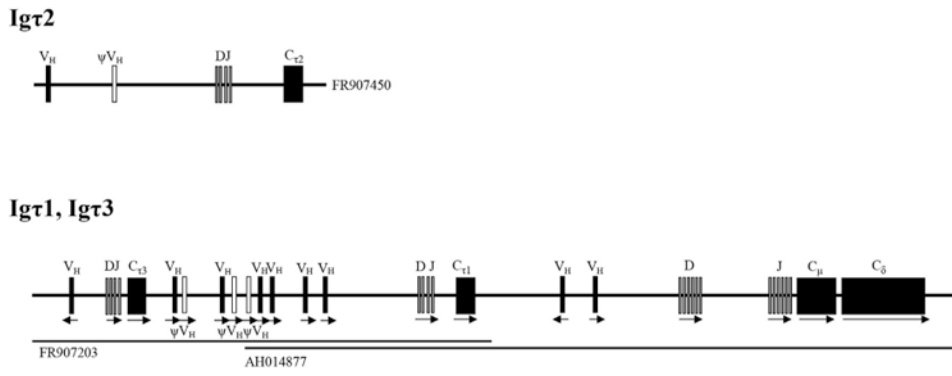


Fig. 4. Genomic organization of rainbow trout IgH loci to scale. Arrow represents the transcriptional orientation. Functional gene segments are marked by filled symbols and pseudogenes (ψ) by open symbols.

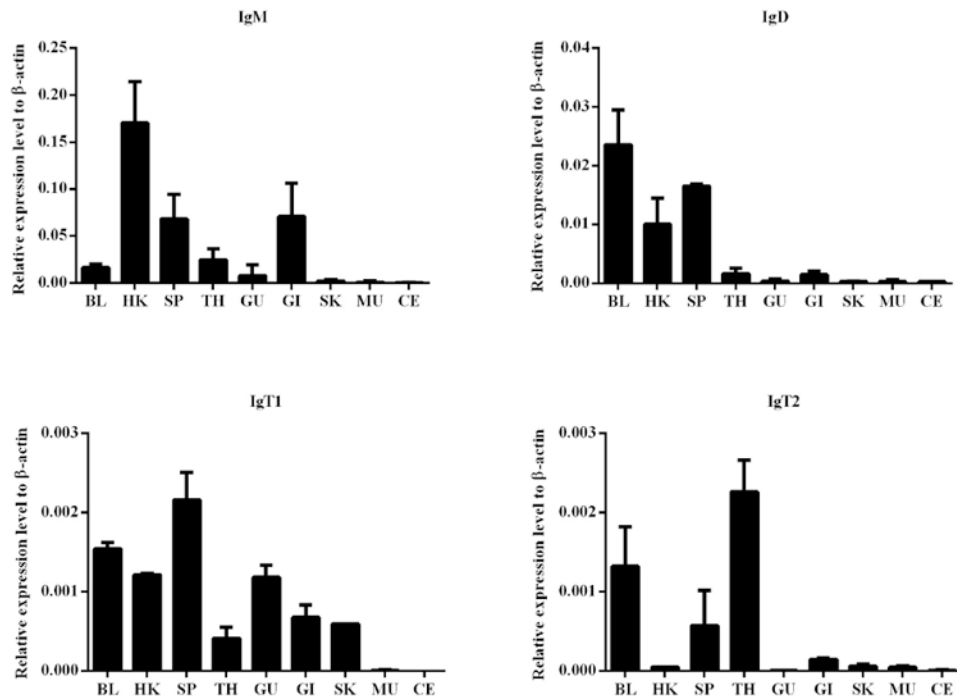


Fig. 5.

Expression patterns of trout IgH genes in various tissues. The relative expression levels were detected by qPCR, and β -actin was used as an internal control. Abbreviations for tissues are as follows: BL, blood; HK, head kidney; SP, spleen; TH, thymus; GU, gut; GI, gill; SK, skin; MU, muscle; CE, cerebrum. The values represent the mean \pm SD of three individual fish.

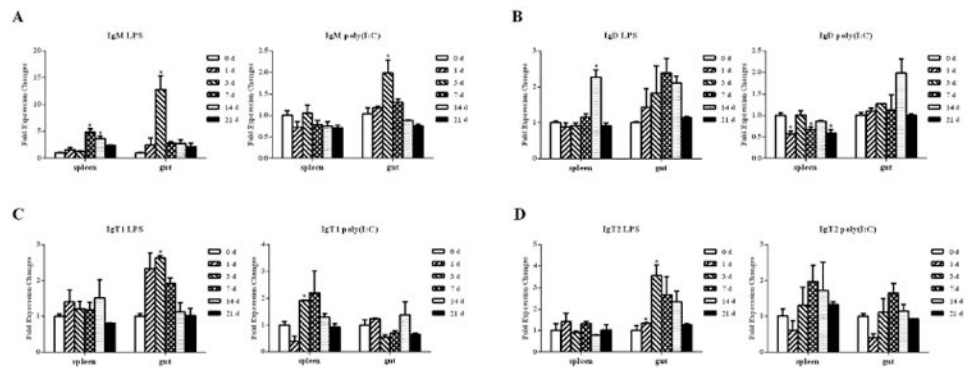


Fig. 6.

Expression analyses of the IgH genes in the spleen and gut of the trout stimulated with LPS or poly (I:C). The relative expression levels were detected by qPCR and normalized against the expression at 0 d (set as 1). EF-1 α and β -actin were used as internal controls. The values represent the mean \pm SD of each individual fish. Asterisks indicate significant differences between each time point and control time point (0 d), the p values were calculated by Student's *t*-test (*, $p < 0.05$).

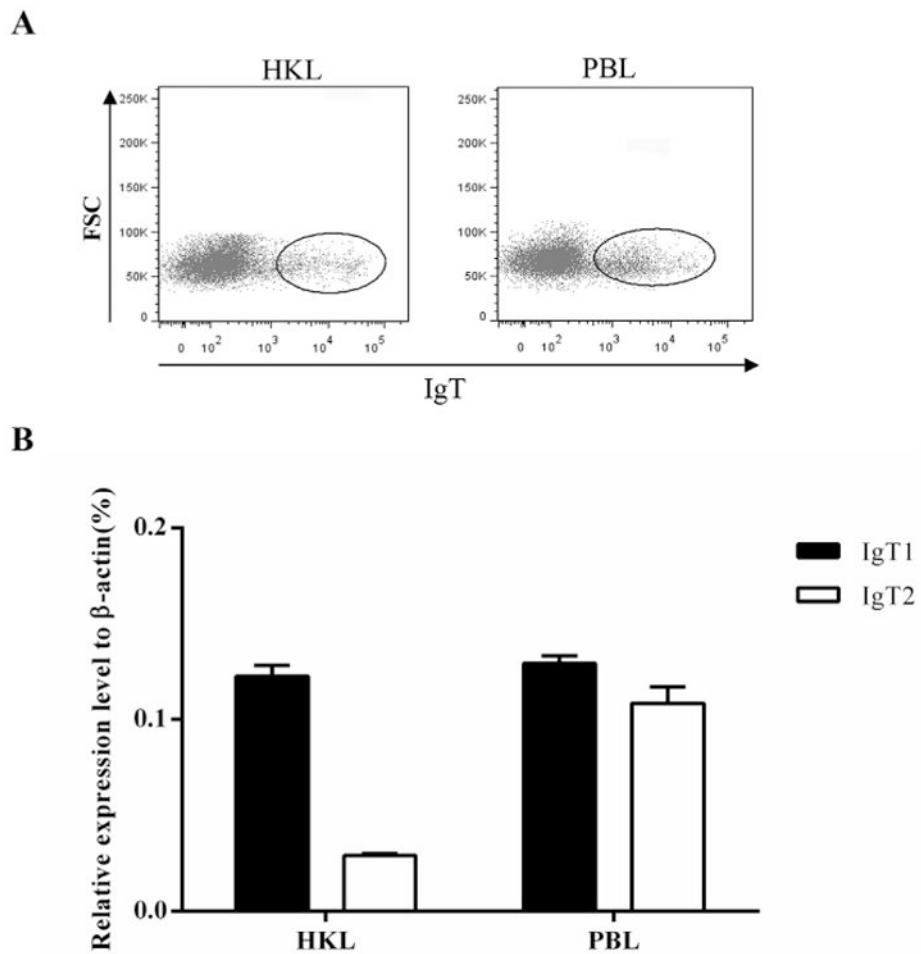


Fig. 7. Expression analyses of trout $Ig\tau$ genes in the sorted IgT^+ B cells. (A) Anti-trout $IgT1$ mAb was used to sort the IgT^+ B cells from HKLs and PBLs. (B) The relative expression levels of trout $Ig\tau1$ and $Ig\tau2$ were detected by qPCR, and β -actin was used as an internal control. The values represent the mean \pm SD of three individual fish.

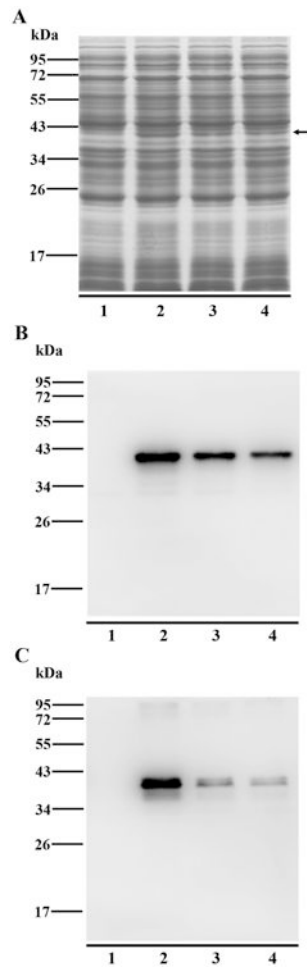


Fig. 8. Western blotting analysis of the reactivity of anti-trout IgT1 mAb to other IgT subclasses. The CH2–CH4 regions of trout IgT1, IgT2 and IgT3 were recombinantly expressed in *E.coli*. Supernatants of the cell lysates (containing the soluble recombinant proteins) were subjected to SDS-PAGE under reducing condition for coomassie blue staining (A) and under both reducing (B) and non-reducing (C) conditions for immunoblotting with mouse anti-trout IgT1 mAb. The total proteins loaded on SDS-PAGE were 15 μ g for gel staining and 5 μ g for immunoblotting. The expressed recombinant proteins were directed by a black arrow. Lane 1: blank control (trout Ig τ 1 without IPTG induction); Lane 2: trout Ig τ 1; Lane 3: trout Ig τ 2; Lane 4: trout Ig τ 3.

Table 1

LC-MS/MS analyses of the IgT subclasses in trout serum.

Peptide	Subclass	Position (aa in CH)	Frequency
KGFSPSSHTFQWTDASGK	τ 1	28–45	60
KTAVINKPVPK	τ 1	92–102	35
KRPSGLYSGSSLLK	τ 1	152–164	88
RPSGLYSGSSLLK	τ 1	152–164	2
KTTSKTEPLTVTLNPPR	τ 1	189–205	42
KTEPLTVTLNPPR	τ 1	193–205	59
KVQCSAMKRGEDTPVIQDISFTK	τ 1	277–299	11
RGEDTPVIQDISFTK	τ 1	285–299	30
KEDSGEYQEGVTSPPQK	τ 1	341–357	4
KGNYFVTSVFTITK	τ 1	360–373	12
KSGEDTPVIQDLSFTK	τ 2	280–295	77
KGFSPSSHTFK	τ 3	28–38	5
KGFSPSSHTFKWTDASGK	τ 3	28–45	61
KRGDVTPVIQDLSFNK	τ 3	284–299	1
RGDVTPVIQDLSFNK	τ 3	285–299	13
KVNSGDYQEGVTSPPQK	τ 3	341–357	1
KGNYSVTSVFTTTK	τ 3	360–373	8
KHLGSDNNTTPQGR	τ 3	387–400	9
KALTDVQYPAVQSGETYTGVSQLR	τ 1, τ 3	45–69 ^{a,c}	39
KVTNTDWNK	τ 1, τ 3	164–173 ^{a,c}	3
KTEPLTVTLNPPSVK	τ 2, τ 3	193–207 ^{b,c}	2
KNVWENSK	τ 1, τ 2, τ 3	72–79 ^{a,b,c}	4
RVSTLTIDQTR	τ 1, τ 2, τ 3	261–271 ^{a,c} 257–267 ^b	21

^aPosition in the CH region of Ig τ 1.^bPosition in the CH region of Ig τ 2.^cPosition in the CH region of Ig τ 3.