Title

Production of two recombinant insulin-like growth factor binding protein-1 subtypes specific to salmonids

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Abstract

Salmonids have four subtypes of insulin-like growth factor binding protein (IGFBP)-1, termed -1a1, -1a2, -1b1 and 1b2, owing to teleost- and a lineage-specific whole-genome duplications. We have previously produced recombinant proteins of masu salmon IGFBP-1a1 and -1b2 and conducted functional analysis. To further characterize salmonid-specific IGFBP-1s, we cloned cDNAs encoding mature proteins of IGFBP-1a2 and -1b1 from the liver of masu salmon (*Oncorhynchus masou*). IGFBP-1a2 and -1b1 shared a 56% amino acid sequence homology whereas their homologies with their counterparts (i.e. -1a1 and -1b2) were 77% and 82%, respectively. We next expressed recombinant masu salmon (rs) IGFBP-1a2 and -1b1 with fusion partners thioredoxin (Trx) and a His-tag using the pET-32a(+) vector system in *Escherichia coli*. Trx.His.rsIGFBP-1s were detected in the insoluble faction, solubilized in a buffer containing urea, and isolated by Ni-affinity chromatography. They were refolded by dialysis and cleaved from the fusion partners by enterokinase. rsIGFBP-1a2 and -1b1 were purified by reversed-phase high performance liquid chromatography. Purified rsIGFBP-1a2 and -1b1 had the ability to bind digoxigenin-labeled human IGF-I on ligand blotting. We then examined the effects of rsIGFBP-1a1, -1a2, -1b1 and -1b2 in combination with human IGF-I on growth hormone (GH) release from cultured pituitary cells of masu salmon. IGF-I alone reduced GH release while the addition of rsIGFBP-1a1, -1b1 or -1b2, but not rsIGFBP-1a2, diminished the suppressive effect of IGF-I. Addition of rsIGFBP-1s without IGF-I had no effect on GH release. These results show that rsIGFBP-1b1, along with rsIGFBP-1a1 and -1b2, inhibits IGF-I action on the pituitary in masu salmon. The lack of the effect by rsIGFBP-1a2 suggests that salmon IGFBP-1 subtypes underwent subfunction partitioning and have different degrees of IGF-inhibitory action.

Keywords

Whole genome duplication; Tetraploidy; Pituitary cells; Growth hormone release; Subfunction partitioning

1. Introduction

Insulin-like growth factor (IGF)-I is a component of the somatotropic axis playing a crucial role in promoting growth (Daughaday and Rotwein, 1989; Le Roith et al., 2001; Ohlsson et al., 2009). IGF-I is mainly produced by the liver and secreted into the blood stream in response to growth hormone (GH) stimulation, and mediates the actions of GH (Daughaday and Rotwein, 1989). Circulating IGF-I in turn forms a feedback loop to regulate, generally by suppressing, GH release from the pituitary gland (Daughaday and Rotwein, 1989; Ohlsson et al., 2009). IGF-I exhibits its actions by binding mainly to the type I receptor on the cell surface. However, IGF-I in the blood is bound to one IGF-binding protein (IGFBP). Because IGFBPs have IGF-binding affinities higher than or equal to that of the receptor, they regulate availability of IGF-I to the receptor (Zapf, 1995; Hwa et al., 1999).

There are six IGFBPs, termed 1–6, in mammals and they either inhibit or potentiate the action of IGF-I, depending on the type, post-translation modification and cellular environment (Jones and Clemmons, 1995; Rajaram et al., 1997; Firth and Baxter, 2002; Bach, 2018). IGFBP-3 is most abundant in mammalian circulation, carrying 70%–80% of IGF-I (Zapf, 1995; Baxter, 2001). IGFBP-1 is another major circulating form and generally inhibitory to IGF-I action by sequestering IGF-I from circulation under catabolic conditions (Lee et al., 1993, 1997; Weatcroft and Kearnery, 2009).

Teleosts could have two paralogs ("a" and "b") of each member of IGFBPs except IGFBP-4 owing to a third round of whole genome duplication (WGD; Ocampo Daza et al., 2011). Some paralogs have been lost in a lineage-specific manner after teleost-specific WGD. For example, zebrafish (*Danio rerio*) have two copies of IGFBP-1, -2, -5 and -6, but only one of IGFBP-3 and none of IGFBP-4 (Ocampo Daza et al., 2011; Allard and Duan, 2018). These paralogs undergo spatiotemporal subfunction partitioning and play vital roles in regulating embryonic development in zebrafish (Kamei et al., 2008; Zhou et al., 2008; Dai et al., 2010; Wang et al., 2009). Despite the possible importance of paralogs of each IGFBP type for development and growth in fish, only a few studies have analyzed their function in postnatal growth (Cleveland et al., 2018; Tanaka et al., 2018).

Salmonids are unique in that they have a large set of gene repertories, including the IGF, IGF-receptor and IGFBP families, owing to autotetraploidization (Macqueen et al., 2013; Lappin et al., 2016; de la Serrana and Macqueen, 2018). Twenty-two *igfbp* genes have been identified in Atlantic salmon (*Salmon salar*), in which up to four subtypes for each *igfbp* exist (ex. *igfbp-1a1*, *-1a2*, *-1b1* and *-1b2*) (de la Serrana and Macqueen, 2018). High retention rates of duplicated genes and a relatively high degree of diversification within a gene repertory suggest this protein family is important for regulating growth through subfunction partitioning or neofunctionalization (Allard and Duan, 2018; de la Serrana and Macqueen, 2018).

IGFBP-1 is the first member identified in the protein family and a major form in circulation (Lee et al., 1993, 1997; Weatcroft and Kearnery, 2009). This form is unique in terms of being unoccupied with endogenous IGFs and showing a dynamic fluctuation in response to meals and stress. Mammalian IGFBP-1 has a Arg–Gly–Ser (RGD) sequence, a motif to bind integrin on the cell surface, in the C-terminus and a segment rich in Pro, Glu, Ser and Thr (PEST domain) in the mid-region that enhances turnover of proteins (Jones et al., 1993; Lee et al., 1993, 1997; Forbes et al., 2012). Phosphorylation is an important post-translational modification of IGFBP-1 because it increases the IGF-binding affinity and thus prevents IGFs from binding to the receptor (Jones et al., 1991). In zebrafish, IGFBP-1 is also inhibitory to IGF-I action, but essential for the survival of embryos under hypoxia by reducing energy expenditure induced by IGF-I (Kajimura et al., 2005; Kamei et al., 2008).

In salmon, IGFBP-1a and -1b are two of three major forms in the circulation, the other being IGFBP-2b (Shimizu et al., 2005, 2011a,b; Shimizu and Dickhoff, 2017), although it is not clear if the blood contains both salmonid-specific paralogs of each subtype. IGFBP-1a and -1b are induced into bloodstream by fasting and stress while IGFBP-1b appears to be induced earlier under those catabolic conditions (Shimizu et al., 2006, 2011a; Kawaguchi et al., 2013). Moreover, quantification of circulating IGFBP-1a and -1b revealed that both are negatively correlated with individual growth rate in masu salmon (*O. masou*; Kaneko et al., 2020). These physiological responses of IGFBP-1a and -1b suggest that they are also inhibitors of the action of circulating IGF-I in salmon.

We previously reported production of recombinant proteins for masu salmon IGFBP-1a1 and -1b2 (Tanaka et al., 2018) using a bacterial expression system. Functional analysis using recombinant IGFBP-1s and a primary pituitary cell culture of masu salmon revealed that IGF-I action on GH release was significantly suppressed by adding IGFBP-1a1 whereas the effect of IGFBP-1b2 was not significant (Tanaka et al., 2018). The findings of the study suggest that IGFBP-1a1 modulates the GH feedback loop by IGF-I and is a more potent inhibitor of IGF-I action than IGFBP-1b2. However, because salmonids have four subtypes of IGFBP-1, there are questions as to whether the two other subtypes possess IGF-inhibitory action and, if so, to what extent. The present study produced recombinant proteins of IGFBP-1a2 and -1b1, and conducted functional analysis together with IGFBP-1a1 and -1b2.

2. Materials and Methods

2.1. Cloning of partial cDNAs encoding mature proteins of masu salmon igfbp-1a2 and -1b1

A liver sample from yearling masu salmon smolt caught in May at the Meppu River, South Hokkaido, Japan was used for cDNA cloning. Total RNA was extracted from the liver using Isogen (Nippon gene; Tokyo, Japan) and single-strand cDNA was reverse-transcribed using

SuperScript III (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions.

Primers were designed based on the *igfbp-1* sequences of Atlantic salmon (Macqueen et al., 2013) to amplify the complete and partial open reading flames (ORFs) of masu salmon *igfbp-1a2* and *-1b1*, respectively (Table 1). Reverse transcriptase (RT)-PCR was performed using AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Foster City, CA) and a Veriti Thermal Cycler (Applied Biosystems). PCRs consisted of 1 cycle of 95°C for 10 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min; 1 cycle of 72°C for 7 min. PCR products were run on a 1.5% agarose gel, purified and cloned into the pGEM-T Easy Vector Systems (Promega, Madison, WI). Positive clones were sequenced as described in Shimizu et al. (2011b).

2.2. Construction of expression vectors and expression of recombinant proteins

Detailed procedures for vector construction and protein expression are described in Tanaka et al. (2018). Briefly, the pET-32a(+) (Novagen, Madison, WI), which adds thioredoxin (Trx) and a His-tag (6 × His) at the N-terminal region of the expressed recombinant protein, was used as an expression vector. The constructed plasmids (pET-Trx.His.rsIGFBP-1a2 and -1b1) were transformed into the *Escherichia coli* strain Rosetta-gami™ B(DE3)pLysS (Novagen). Expression of Trx.His.rsIGFBP-1a2 and -1b1 was induced by adding isopropyl-β-D-thiogalactoside (IPTG). Bacterial proteins were separated into soluble and insoluble fractions by using Bugbuster Protein Extraction Reagent (Novagen). The insoluble fractions containing recombinant proteins were solubilized by urea and subjected to His-Bind Resin (Novagen) chromatography. The eluate from the column was dialyzed against 20 mM Tris-HCl (pH 8.0) containing 2% NaCl and urea was removed stepwisely to facilitate correct refolding.

2.3. Enzymatic digestion and purification of recombinant proteins

After dialysis, the fusion partners (i.e. Trx.His) were cleaved by an enterokinase using a Tag.off™ High Activity rEK Kit (Novagen). Twenty micrograms of protein was mixed with 1 µl (1 unit) enterokinase and incubated at room temperature for 20 h. rsIGFBP-1a2 or -1b1 were further purified by reverse-phase high performance liquid chromatography (HPLC) using a Vydac C-4 column (0.46 × 5 cm; Separation Group, Hesperia, CA) as described in Tanaka et al. (2018).

2.4. Electrophoresis, ligand blotting and western blotting

Recombinant proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 3% stacking gel and 12.5% separating gel. Samples were treated with an equal volume of the sample buffer containing 2% SDS and 10% glycerol with or without 5% 2-mercaptoethanol at 85°C for 5 min. Gels were run at 50 V in the stacking gel and at 100 V in the separating gel, and were stained with 0.1% Coomassie Brilliant Blue (CBB) R250

(Bio-Rad, Hercules, CA). Molecular mass was estimated with Precision Marker (Bio-Rad).

IGF-binding ability of the recombinant proteins was assessed by western ligand blotting with digoxigenin-labeled human IGF-I (DIG-hIGF-I) as described in Shimizu et al. (2000). After electrophoresis, proteins were electroblotted onto a nitrocellulose membrane. The membrane was incubated with 50 ng/ml DIG-hIGF-I for 2 h at room temperature and then with an antibody against DIG conjugated with horseradish peroxidase (Roche, Indianapolis, IN) at a dilution of 1:2,000 for 1 h at room temperature. IGFBP bands were visualized using enhanced Chemiluminescence (ECL) Prime western blotting reagents (Amersham Life Science, Arlington Heights, IL) and ImageQuant LAS 500 (GE Healthcare, Uppsala, Sweden).

For western blotting, 100 ng of purified recombinant proteins were separated by 12.5% SDS-PAGE and electroblotted on to an nitrocellulose membrane. The membrane was incubated with antiserum against rsIGFBP-1a1 (Kaneko et al., 2020) at a dilution of 1:2,000 or antiserum against purified Chinook salmon (*O. tshawytscha*) IGFBP-1b (Shimizu et al., 2006) at a dilution of 1:2,000 for 2 h at room temperature. The membranes were then probed with goat anti-rabbit IgG-HRP conjugate (Bio-Rad) at a dilution of 1:10,000 for 1 h at room temperature. Immunoreactive bands were visualized using the ECL reagents as described above.

2.5. Primary pituitary cell culture

Primary pituitary cell culture was conducted according to the methods of Ando et al. (2004) with modifications as detailed in Tanaka et al. (2018). For an initial experiment, pituitaries were collected from 40 2-year-old nonmaturing masu salmon of both sexes (fork length 31.8 ± 0.4 cm, body weight 376.4 ± 13.0 g; expressed as mean ± SE of 10 fish) at Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University (Abuta-gun, Hokkaido, Japan) in January 2015. The sampling was carried out in accordance with guidelines of the Hokkaido University Animal Care and Use Committee (Approval No. 30-3). Pituitaries were minced and digested with 0.1% collagenase at 12°C for 20 h. The dispersed cells were plated on 12-well culture plates (BD Biosciences, Franklin Lakes, NJ), pre-coated with 50 μg/ml poly-D-lysine hydrobromide (Sigma, St. Louis, MO), at a density of 2.3×10^5 cells/1.3 ml medium/well ($n = 3$) and incubated in the RPMI medium supplemented with Serum Replacement 2 (Sigma-Aldrich, Tokyo, Japan) at 12°C for 48 h. Recombinant salmon IGF-I (sIGF-I; GroPep Bioreagents, Thebarton, Australia) and hIGF-I (GroPep) were added to the medium to make each final concentration of 1, 10 or 100 nM and the cells were incubated for additional 24 hr.

Another batch of pituitaries was collected in late November 2019 from 70 1-year-old nonmaturing masu salmon of both sexes (fork length 21.8 ± 1.2 cm, body weight 116.6 ± 19.7 g; expressed as mean ± SE of 10 fish) and treated as described above. rsIGFBP-1a1, -1a2, -1b1 and -1b2 were added to the medium with or without hIGF-I to make each final concentration of 100

nM and the cells were incubated for additional 24 hr.

2.6. Time-resolved fluoroimmunoassay (TR-FIA) for GH

GH levels in medium were quantified by TR-FIA. A competitive method was employed by following a procedure for DELFIA immunoassays (PerkinElmer, Waltham, MA). Purified chum salmon GH and antiserum against purified chum salmon GH were gifts from Shunsuke Moriyama and Akihiko Hara, respectively. Purified chum salmon GH was biotinylated according to the method described in Fukuda et al. (2015). Forty microliters of GH standard or medium samples diluted with DELFIA Assay Buffer were first incubated with 40 µl antiserum against purified chum salmon GH (1:32,000) overnight at 4°C in a 96-well microtiter plate coated with goat anti-rabbit IgG (PerkinElmer). Forty microliters of biotinylated purified chum salmon GH (1.4 ng) was added to each well and incubated overnight at 4°C. After washing with DELFIA Wash Buffer (PerkinElmer), each well received 160 µl Eu-labeled streptavidin (1:2,000; PerkinElmer) followed by DELFIA Enhancement Solution (PerkinElmer). Time-resolved fluorescence was measured using the Wallac ARVO X4 multilabel counter (PerkinElmer). Measured values were expressed as the % of control cells treated with neither hIGF-I nor rsIGFBP-1.

2.7. Statistical analysis

Results of the cell culture experiments were analyzed by one-way ANOVA using the JMP program (SAS Institute Inc., Cary, NC). When significant effects were found, differences were further identified by Fisher's protected least significant difference (PLSD) test. Differences between groups were considered significant at $P < 0.05$.

3. Results

3.1. Cloning of partial cDNAs encoding mature proteins of masu salmon igfbp-1a2 and -1b1

cDNAs encoding mature proteins of masu salmon *igfbp-1a2* and *-1b1* were cloned and their sequences were deposited in GenBank (Accession Nos. MT010531 and MT010532). The estimated molecular weights of mature masu salmon IGFBP-1a2 and -1b1 were 23.9 kDa and 23.8 kDa with 222 and 219 amino acid residues, respectively. They possessed the 18 cysteine residues conserved in the IGFBP family (Fig. 1). Amino acid sequence homologies of masu salmon IGFBP-1a2 and -1b1 with those of Atlantic salmon were 90% and 95%, respectively. There were >77% sequence homologies within the subtype (i.e. within "a"- or "b"-subtype) and >56% between the subtypes (Table 2).

3.2 Expression and purification of rsIGFBP-1a2 and -1b1

A band at 41 kDa was visible in the extracts of the bacterial cells transformed with pET-32a-

Trx.His.IGFBP-1a2, which became more dense after addition of IPTG (Fig. 2a). The 41-kDa band was exclusively found in the insoluble fraction. A band at 41 kDa was also induced by IPTG in the bacterial cells transformed with pET32a-Trx.His.IGFBP-1b1 (Fig. 2b). The induced protein was mainly fractionated into the insoluble fraction. These recombinant proteins with His-tag were solubilized by urea and affinity-purified using a Ni-column (data not shown). After refolding by dialysis, Trx.His.rsIGFBP-1a2 and -1b1 bands at 35 kDa under nonreducing conditions were capable of binding DIG-IGF-I on ligand blotting (Fig. 3). Trx and His-tag were removed from rsIGFBP-1a2 and -1b1 by enterokinase (Fig. 3). Both Trx.His.IGFBP-1s were cleaved into 20-kDa bands which retained the ability to bind hIGF-I. Under the experimental conditions, the 35 kDa bands were still visible, although increasing the amount of enterokinase resulted in degradation of the 20-kDa bands (data not shown). rsIGFBP-1a2 and -1b1 without fusion partners were purified by reversed-phase HPLC (Fig. 4). Purified rsIGFBP-1a2 was detected as a primary band at 20 kDa on SDS-PAGE under nonreducing conditions and rsIGFBP-1b1 also appeared at the same position (Fig. 5). Minor bands were detected at higher-molecular-weights in purified rsIGFBP-1a2 and -1b1 (Fig. 5). On immunoblotting, rsIGFBP-1a2 showed little cross-reactivity with anti-rsIGFBP-1a1 (Fig. 6). rsIGFBP-1b1 and -1b2 were both visualized by anti-Chinook salmon IGFBP-1b, while IGFBP-1b1 showed a higher cross-reactivity (Fig. 6).

3.3 Effects of IGF-I and rsIGFBP-1 subtypes on GH release from pituitary cells

Effects of sIGF-I and sIGF-I on GH release from cultured masu salmon pituitary cells were first examined (Fig. 7). Both IGFs reduced GH release when added at final concentrations of 10 and 100 nM, and the maximum inhibition was achieved at 100 nM. Given the limitation of quantity of commercially available sIGF-I, hIGF-I was used for further analysis. Effects of four rsIGFBP-1s with or without hIGF-I on GH release from cultured masu salmon pituitary cells were then examined (Fig. 8). Addition of hIGF-I at 100 nM suppressed GH release by 24 h (Fig. 8a). When 100 nM rsIGFBP-1a1, -1b1 and -1b2 were added in combination with hIGF-I, GH release in those treatments became higher than that with IGF-I alone and was restored to the control levels. On the other hand, addition of rsIGFBP-1a2 with hIGF-I did not significantly increase GH release when compared to the treatment with hIGF-I alone. None of rsIGFBP-1 affected the GH release without hIGF-I (Fig. 8b).

4. Discussion

Salmonids have an exceptionally large number of *igfbp* genes (i.e., 22 genes) that are products of autotetraploidization (Macqueen et al., 2013; de la Serrana and Macqueen). Conservation of the duplicated IGFBP family members suggests that fine tuning of the IGF-I signal by IGFBP subtypes had a selective advantage (Allard and Duan, 2018). The presence of four subtypes for

each IGFBP family member except IGFBP-4 has been reported in Atlantic salmon and rainbow trout (*O. mykiss*; Macqueen et al., 2013; de la Serrana and Macqueen, 2018). However, no study has compared the activity of the four subtypes of a single IGFBP. We produced two recombinant proteins for IGFBP-1a2 and -1b1, in addition to IGFBP-1a1 and -1b2 (Tanaka et al., 2018), and performed functional analysis. Our results suggest that at least three of four IGFBP-1 subtypes are inhibitory to IGF-I action on the pituitary.

Two cDNAs for masu salmon *igfbp-1*s have been cloned by Tanaka et al. (2018) and the present study cloned two other cDNAs encoding mature proteins of *igfbp-1* subtypes, confirming that this species expresses four *igfbp-1*s. The identity as *igfbp-1b1* of one of two cDNAs cloned in the present study was unexpected; we assumed that the previously cloned masu salmon *igfbp-1b* (Tanaka et al., 2018) was *igfbp-1b1* given that its deduced N-terminal 19-amino acid sequence was identical to those of Atlantic and Chinook salmon *igfbp-1b1*s. Cloned *igfbp-1a2* and *-1b1* had 18 cysteine residues in both termini, which are conserved in the IGFBP family and necessary for high binding affinity for IGFs (Forbes et al., 2012; Allard and Duan, 2018). A Pro (P), Glu (E), Ser (S) and Thr (T)-rich segment called PEST domain, characteristic of rapidly metabolized protein including human IGFBP-1 (Julkunen et al., 1988; Lee et al., 1997), was detected in the mid-region of masu salmon IGFBP-1a2, although the score for a potential PEST motif (epestfind; http://emboss.bioinformatics.nl/cgi-bin/emboss/epestfind) was rather low (data not shown). No Arg–Gly–Ser (RGD) sequence was present in the C-terminus, which is in contrast to mammalian orthologs but agrees with salmon paralogs (Shimizu et al., 2011a). Although there are differences in the protein motifs compared with mammalian IGFBP-1, an inhibitory action of the four IGFBP-1 subtypes in salmonid have been proposed (Shimizu and Dickhoff, 2017). de la Serrana and Macqueen (2018) predicted protein complexes of IGF–IGFBP using a modeling pipeline and revealed that the N-terminal region of IGFBP-1 paralogs had more contacts with IGFs than those of IGFBP-3 and -5, enabling them to compete with the IGF-receptor and thus inhibit IGF action.

Despite the conserved basic protein structure of salmon IGFBP-1s, there were certain differences in amino acid sequences among the four subtypes. Sequence homologies of deduced amino acids of mature IGFBP-1 between "a" and "b" types and between "type 1" and "type 2" in masu salmon were 56%–63% and 77%–82%, respectively, which is in accord with the finding in Atlantic salmon (Macqueen et al., 2013). Given that an average of 93% amino acid identity is found across thousands of paralog pairs in rainbow trout (Berthelot et al., 2014), the amino acid sequences of the four IGFBP-1 paralogs are considerably different, which supports the notion that duplicate IGFBPs are functionally divergent (de la Serrana and Macqueen, 2018).

The bacterial expression system used in Tanaka et al. (2018) to produce other subtypes of masu salmon IGFBP-1 with fusion partners (i.e., Trx and His-tag) was effective for IGFBP-1a2 and -1b1. Expressing with Trx was not effective at solubilizing the recombinant proteins but

might help refold them. However, a difficulty in this system was low recovery of rsIGFBP-1 from digestion by enterokinase to cleave the fusion partners. Enterokinase is a serine protease targeting the sequence $(Asp)_4Lys$ (Anderson et al., 1977). It is highly specific to the sequence, which does not exist in IGFBP-1a2 or -1b1. However, degradation of rsIGFBP-1a2 and -1b1 occurred. Unwanted cleavage of the recombinant protein by enterokinase has been observed because of its proteolytic activity on other sites and to protein aggregation affecting its accessibility (Shahravan et al., 2008; Gasparian et al., 2011). Optimizing the reaction conditions or/and modifying the target sequence may improve the recovery of rsIGFBP-1a2 and -1b1 after digestion by enterokinase. Although improvement of the procedure is still necessary, the expression system employed in the present study is applicable for producing recombinant proteins for other members of the IGFBP family

A negative feedback loop by IGF-I on GH synthesis and release from the pituitary gland is an essential component of the somatotropic system (Ohlsson et al., 2009). In vitro studies using cultured pituitary cells from teleosts have shown the negative effect of hIGF-I on GH release (Pérez-Sánchez et al., 1992; Fruchtman et al., 2000, 2002; Kajimura et al., 2002). The present study also confirmed that hIGF-I inhibited GH release from pituitary cells of 2- and 1-year-old immature masu salmon of both sexes. However, it should be noted that we previously reported that addition of hIGF-I increased GH release in a similar experiment (Tanaka et al., 2018). We have no good explanation for the difference but the fact was that fish used in Tanaka et al. (2018) had lower condition factor (i.e. skinnier) than those used in the present study, suggesting that feeding condition of fish affected the sensitivity to IGF-I of the pituitary cells. Examining the state-dependent response of the pituitary cells to IGF-I in masu salmon is a subject of future study.

The present study showed that three of the four IGFBP-1 subtypes were capable of inhibiting the IGF-I action on GH release from the pituitary cells. As mentioned above, hIGF-I alone suppressed GH release. However, when equal mols of rsIGFBP-1s were added together with hIGF-I, the IGF-I action was inhibited. Such an IGF-inhibitory action by rsIGFBP-1a1 was reported in Tanaka et al. (2018), but the present study is the first to show that three of the four subtypes (i.e. -1a1, -1b1 and -1b2) inhibited the IGF-I action on GH release. In the previous study, rsIGFBP-1b2 was not effective at inhibiting IGF-I action (Tanaka et al., 2018). Such difference in the effect again suggests that the sensitivity of pituitary cells differed between the two trials. The finding that rsIGFBP-1a2 was not effective at diminishing IGF-I action suggests that the degree of inhibitory action differs among the four subtypes. Our observation is in line with the finding by Kamei et al. (2008) that zebrafish IGFBP-1a and -1b were both inhibitory to IGF-I-induced cell proliferation in cultured zebrafish embryonic cells but IGFBP-1a was more potent than IGFBP-1b.

We used hIGF-I instead of sIGF-I to compare the IGF-modulatory activity of rsIGFBP-

1s. hIGF-I has been used to examine its effects on the synthesis and release of pituitary hormones in salmonids (Pérez-Sánchez et al., 1992; Furukuma et al., 2008; Luckenbach et al., 2010) because of a >80% sequence homology between hIGF-I and sIGF-I. In addition, amino acid residues critical for binding to IGFBPs such as Glu3 and Phe49 (Clemmons et al., 1992; Denley et al., 2005) and those for the IGF-I receptor such as Tyr24 and Tyr60 (Bayne et al., 1990; Denley et al., 2005) are conserved between hIGF-I and sIGF-I. In the present study, hIGF-I was as effective as sIGF-I to suppress GH release from cultured masu salmon pituitary cells, while sIGF-I showed a clearer dose-dependent effect on GH release. Given the limited amount of commercially available sIGF-I, which has been out of stock for five years, hIGF-I was chosen to look at the activity of rsIGFBP-1. Another notable thing is that the doses of hIGF-I and rsIGFBP-1s added to the pituitary culture (100 nM) were higher than their physiological ranges in masu salmon (approximately 10 nM; Kaneko et al., 2020). Thus, dose-dependency of the IGFBP effects within physiological ranges needs to be addressed in future study. The high dose of hIGF-I was chosen to assure inhibition of GH release by hIGF-I, which might be influenced by season and stage/condition of fish, and the same dose of rsIGFBP-1 was added to make the molar ratio 1:1. For detailed functional analyses of IGF-I and IGFBP-1s in salmon, the use of a homologous IGF-I as well as IGFBP-1s at concentrations covering physiological ranges is desired, which, however, would require re-establishment of production of rsIGF-I.

The assumption that IGFBP-1 subtypes of salmon are inhibitory to IGF-I action and thus growth is also supported by their inverse relationships with growth rate. Immunoassays for salmon IGFBP-1a and -1b have been established (Shimizu et al., 2006; Fukuda et al., 2015; Kaneko et al., 2020). Especially, Kaneko et al. (2020) used rsIGFBP-1a1 as an antigen to generate antiserum and an assay standard. Correlation analyses revealed that serum IGFBP-1a and -1b were both inversely correlated with individual growth rate in masu salmon (Kaneko et al., 2020). These findings suggest that circulating IGFBP-1a and -1b are physiologically inhibitory to the IGF-I-mediated growth and useful as indices of growth retardation.

It is worth noting that polyclonal antiserum raised against purified Chinook salmon IGFBP-1b cross-reacted with both rsIGFBP-1b1 and -1b2 while rsIGFBP-1b1 showed a stronger band on immunoblotting. The result of immunoblotting suggests that the purified protein used as antigen was IGFBP-1b1. This also implies that the TR-FIA using the antiserum against IGFBP-1b (Fukuda et al., 2015) measures IGFBP-1b2 to some extent if it is present in the circulation. Indeed, minor bands besides the three major IGFBPs in salmon and trout blood are often detected by ligand blotting (Shimizu and Dickhoff, 2017), one of which could be IGFBP-1b2. Developing/preparing a specific antiserum against each subtype is important to further investigate physiological regulation of IGFBP-1b subtypes and their correlations with individual growth rate. On the other hand, antiserum against rsIGFBP-1a1 showed little cross-reactivity with rsIGFBP-

1a2, suggesting that the TR-FIA for salmon IGFBP-1a (Kaneko et al., 2020) is specific to IGFBP-1a1.

Availability of recombinant proteins for subtypes of each IGFBP in salmon would greatly facilitate functional analysis, especially when combined with gene editing. Cleveland et al. (2018) disrupted the expression of both IGFBP-2b1 and -2b2 by using a CRISPR/Cas9 system in rainbow trout, which resulted in the reduction of circulating levels of both paralogs depending on the mutation rate in F0 fish. The same approach co-targeting IGFBP-1a1/-1a2 or -1b1/-1b2 is possible, and "rescuing" the function of disrupted genes by adding recombinant IGFBP-1s provides a good experimental model to further unravel the role of each IGFBP-1 subtype and the compensatory responses to the loss of an IGFBP in whole fish. Producing other members of salmon IGFBPs and analyzing their roles in growth regulation are also subjects of future research.

In conclusion, we prepared four recombinant masu salmon IGFBP-1 subtypes using a bacterial expression system. IGFBP-1a1, -1b1 and -1b2 diminished the suppressive effect of IGF-I on GH release from the cultured pituitary cells, indicating that they are inhibitors of IGF-I action. The lack of effect by IGFBP-1a2 suggests that the degree of IGF-inhibitory action differs among IGFBP-1 subtypes.

5. Acknowledgments

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540

Figure legends

Fig. 1. Alignment of deduced amino acid sequences of cloned masu salmon IGFBP-1s with those of human and Atlantic (Atl.) salmon IGFBP-1s. Amino acid sequences of human IGFBP-1 (NP_000587.1), Atl. salmon IGFBP-1s (JX565543, JX565544, JX565545 and JX565546) and masu salmon IGFBP-1s (KY471635 and KY471635) were obtained from GenBank. They were aligned by Clustal W method. A putative N-terminal amino acid of mature salmon IGFBP-1s is indicated by an arrowhead. The cysteine residues conserved in the IGFBP family are asterisked. The position of Arg-Gly-Asp (RGD) integrin recognition sequence is underlined. A Pro, Glu, Ser and Thr-rich segment, PEST domain, is in dotted-lined box.

Fig. 2. SDS-PAGE of extracts of bacterial cells transformed with pET32a-Trx.His.IGFBP-1a2 (a) and -1b1 (b) with (+) and without (-) addition of isopropyl-β-D-thiogalactoside (IPTG). Expressed bacterial proteins after addition of IPTG were fractionated into the soluble and insoluble fractions and separated in 12.5% gels under reducing conditions. Migration positions of the molecule weight markers are indicated by arrows on the left. Arrows on the right indicate induced recombinant fusion proteins with molecular weight.

Fig. 3. Enzymatic cleavage of Trx.His.rsIGFBP-1a2 (a) and Trx.His.rsIGFBP-1b1 (b). Twenty micrograms of recombinant proteins were digested by entrokinase (EK) for 20 h, subjected to 12.5 % SDS-PAGE under nonreducing conditions and stained with Coomassie Brilliant Blue (CBB; left panels). The same samples were also subjected to ligand blotting (LB; right panel) using digoxigenin-labeled human IGF-I (50 ng/ml) and antiserum against digoxigenin (1:2,000). Migration positions of molecule weight markers are indicated by arrows on the left. Arrows on the right indicate digested and undigested proteins with molecular weight.

Fig. 4. Purification of rsIGFBP-1a2 (a) and rsIGFBP-1b1 (b) by reversed-phase HPLC. The digested sample (S) was applied to a Vydac C-4 column and eluted by a linear gradient of 0–80 % acetonitrile in 0.1 % trifluoroacetic acid (TFA; dashed lines). Major peaks were collected and analyzed by ligand blotting (insets). Arrows on the ligand blotting indicate migration positions of undigested (35 kDa) and digested (20 kDa) rIGFBP-1a2 (a) and -1b1 (b).

Fig. 5. SDS-PAGE and ligand blotting of purified rsIGFBP-1a2 and -1b1. One-point-three micrograms of purified protein was separated by 12.5% SDS-PAGE under nonreducing conditions (left), followed by analysis by ligand blotting (right) using digoxigenin-labeled human IGF-I (50 ng/ml) and antiserum against digoxigenin (1:2,000). An arrow indicates migration positions of purified proteins.

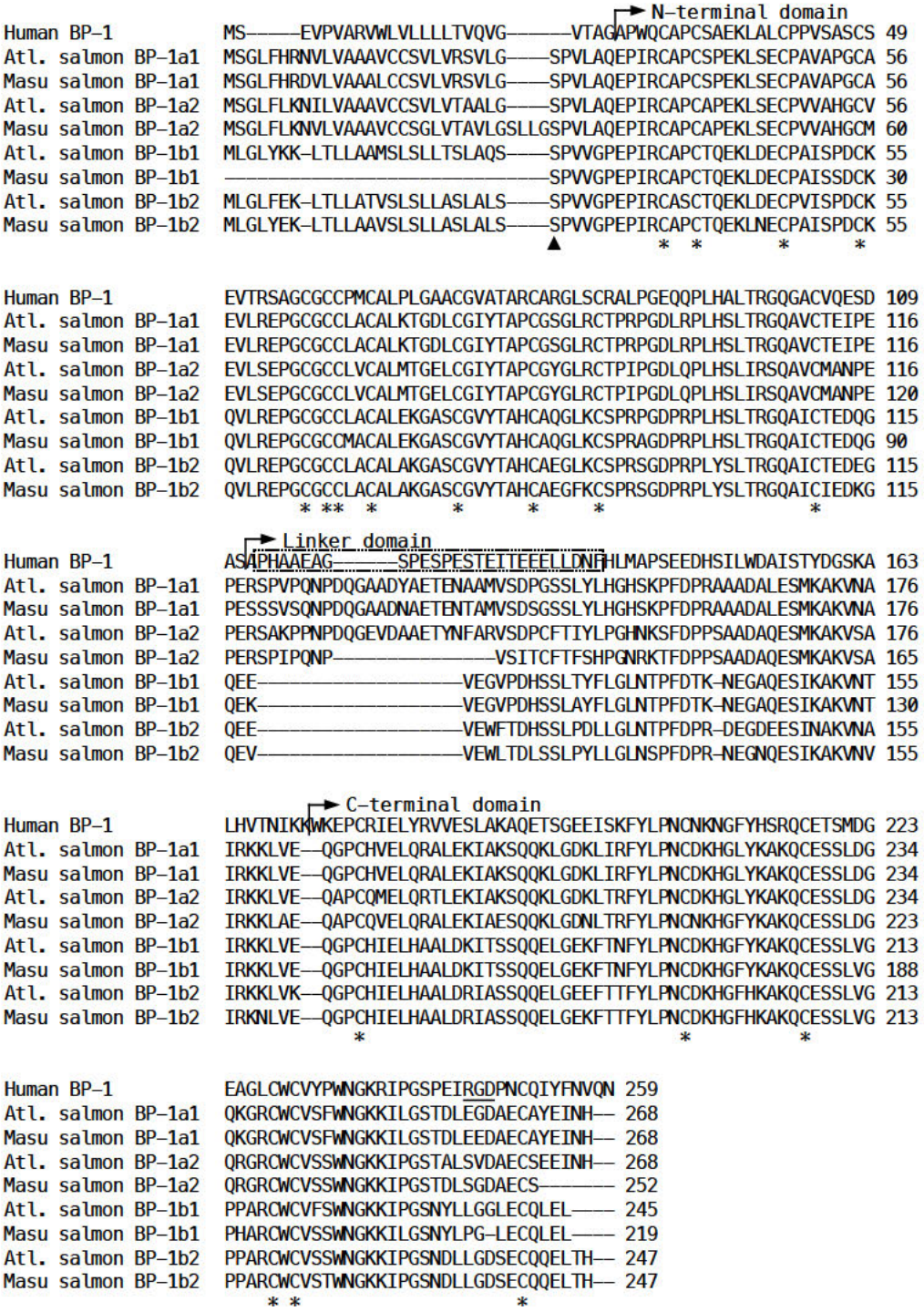
Fig. 6. Immunoblotting of rsIGFBP-1s using antisera against rsIGFBP-1a1 (left panel) and purified Chinook salmon IGFBP-1b (right panel). One hundred nanograms of each rsIGFBP-1 was loaded onto 12.5% gels and electrophoresed under non-reducing conditions. The gels were electroblotted onto nitrocellulose membranes and incubated with anti-IGFBP-1a1 (1:2,000) or anti-IGFBP-1b (1:2,000). The membranes were then proved with anti-rabbit IgG conjugated with horseradish peroxidase (1:5,000) and bands were visualized by the enhanced chemiluminescent reagent.

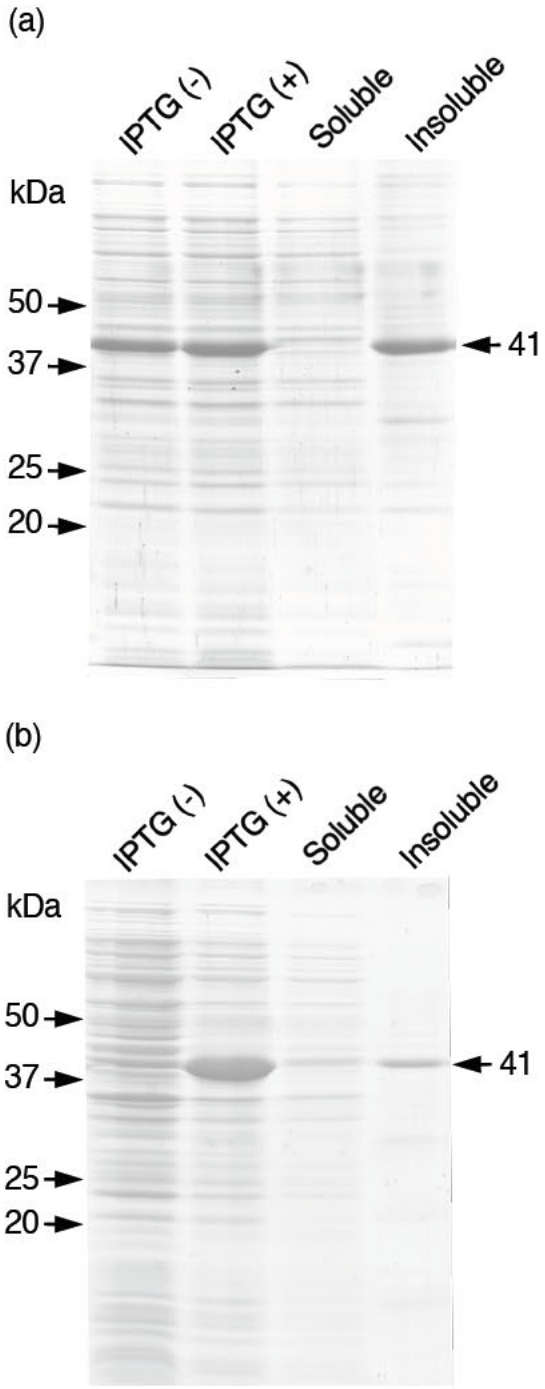
Fig. 7. Effects of salmon (s) and human (h) IGF-Is on GH release from cultured masu salmon pituitary cells. Pituitary cells from two-year-old nonmaturing male and female masu salmon were pre-cultured for 2 days and incubated for 24 h with 1, 10 or 100 nM sIGF-I or hIGF-I. Symbols sharing the same letters are not significantly different from each other (n = 3). C: Control (no treatment with IGF-I).

Fig. 8. Effects of addition of rsIGFBP-1s in the presence (a) and absence (b) of human (h) IGF-I on GH release from cultured masu salmon pituitary cells. Pituitary cells from one-year-old nonmaturing male and female masu salmon were pre-cultured for 2 days and incubated for 24 h without (-) or with (+) 100 nM hIGF-I and 100 nM rsIGFBP-1s. Symbols sharing the same letters are not significantly different from each other (n = 3). Control cells were treated neither hIGF-I nor IGFBP-1.

Figure

Hasegawa et al., Fig. 1

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IPTG (+)
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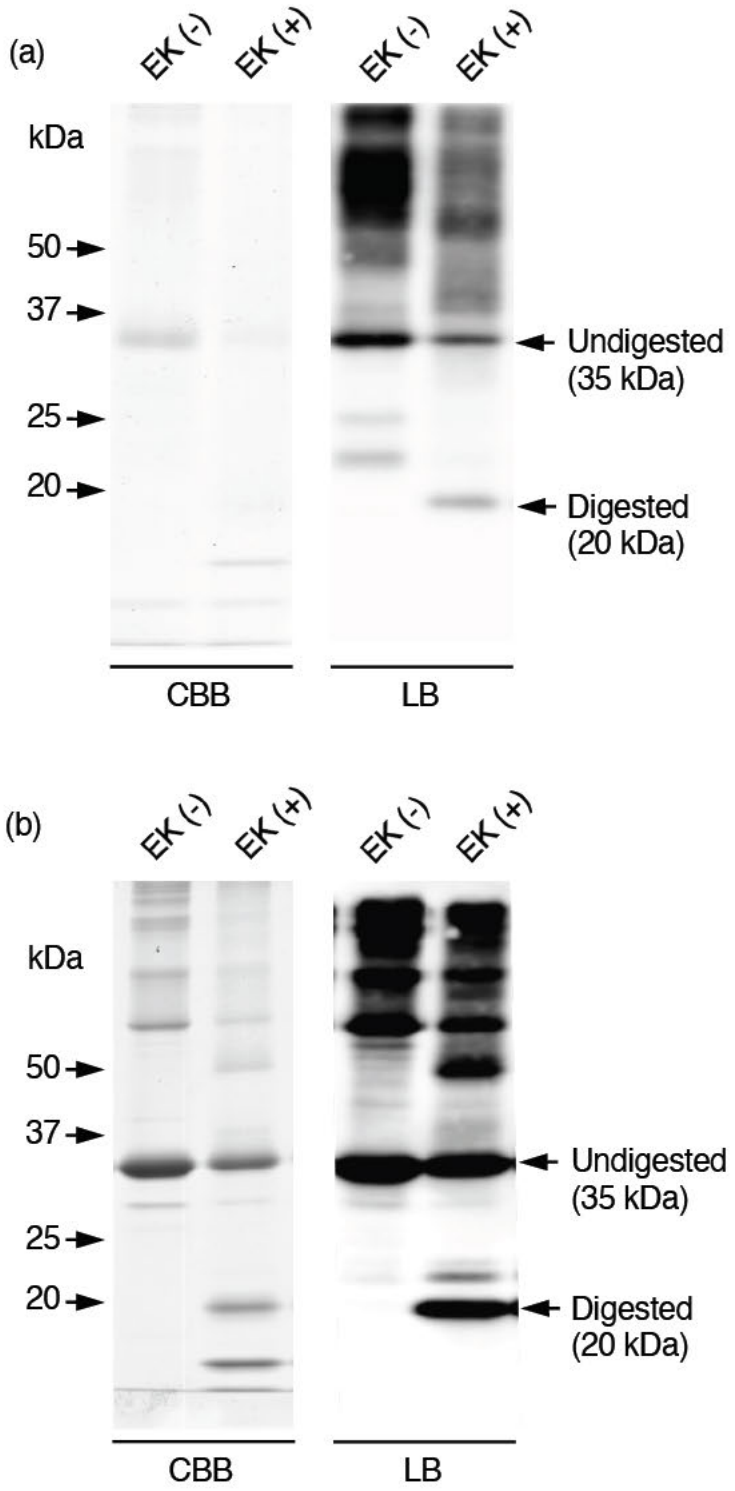
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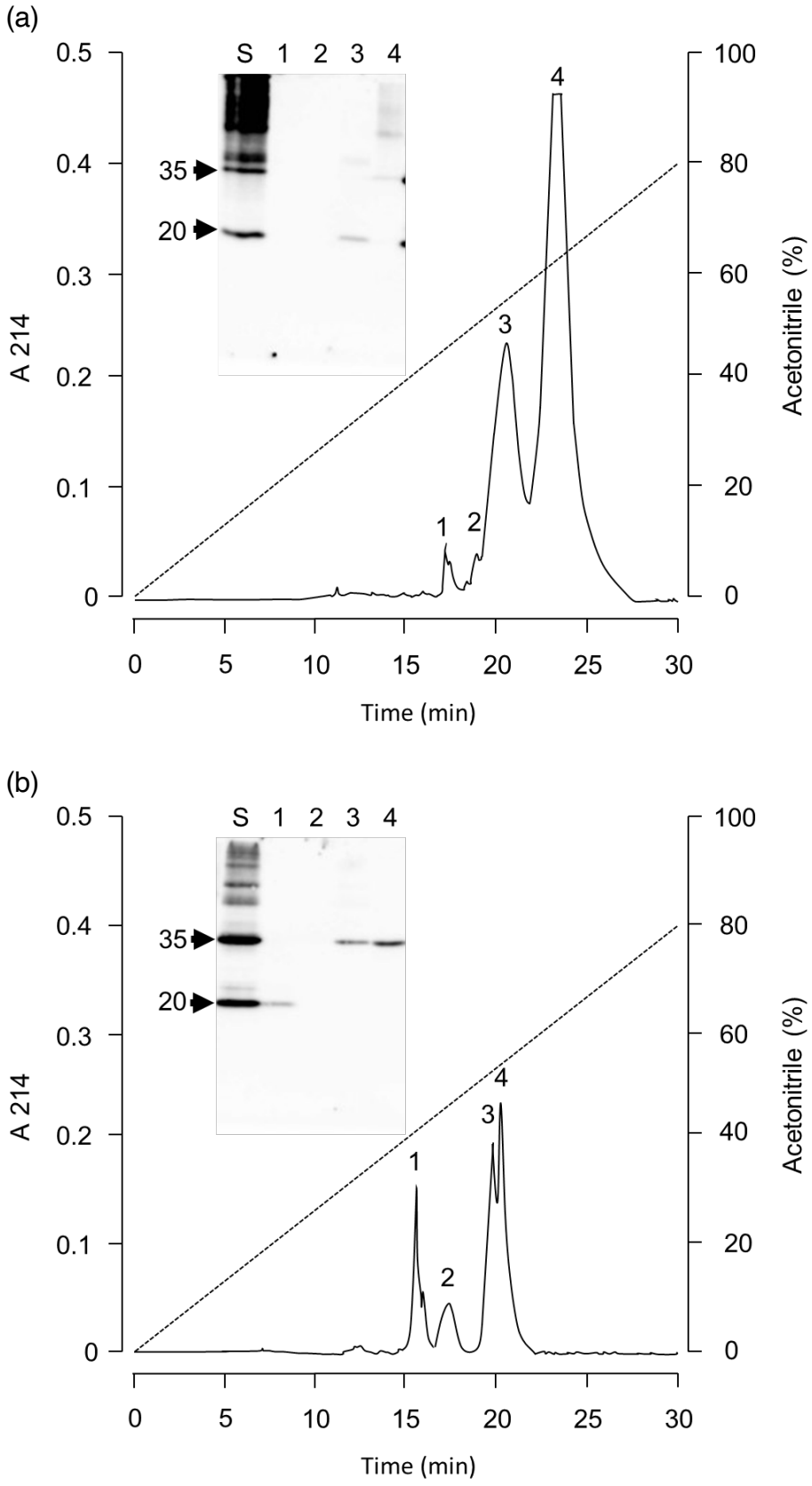
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35
20
A 214
Acetonitrile (%)
Time (min)
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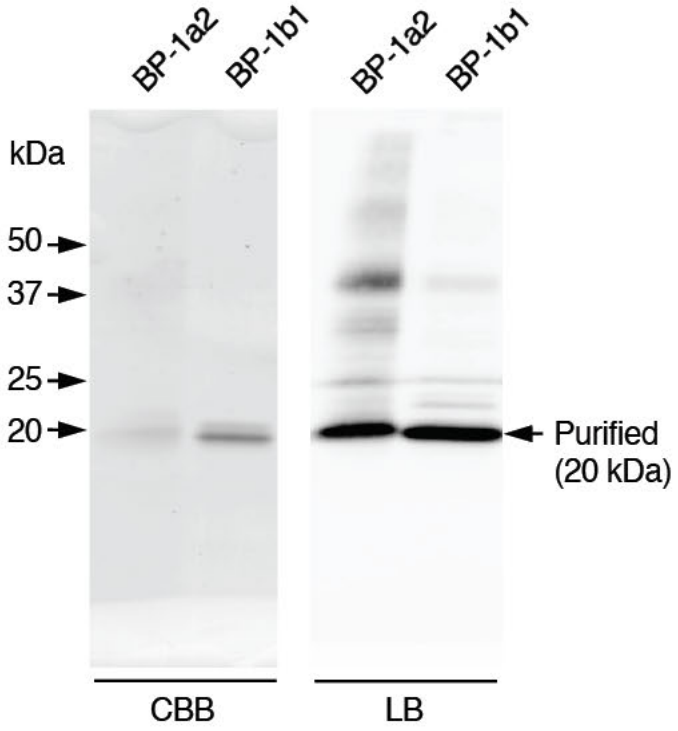
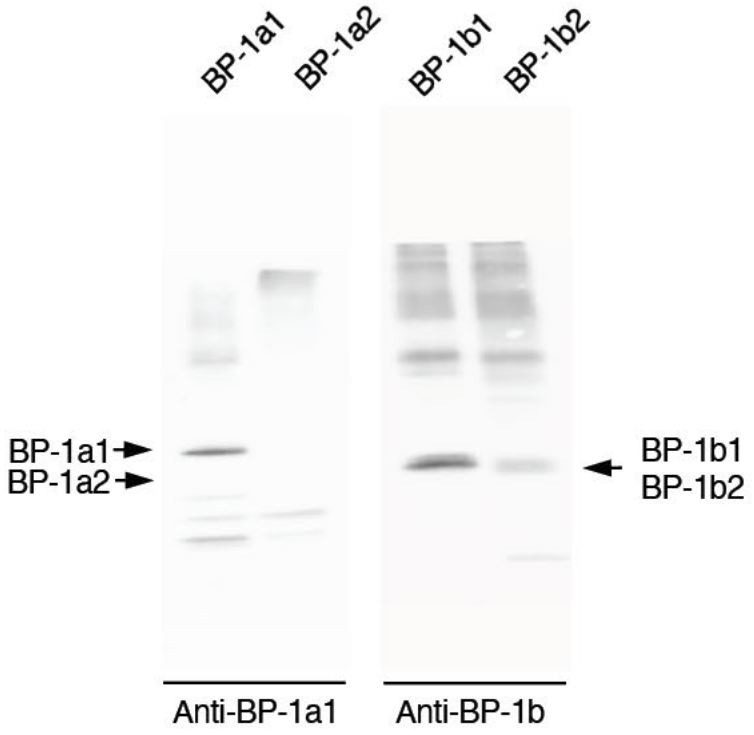
Time (min)

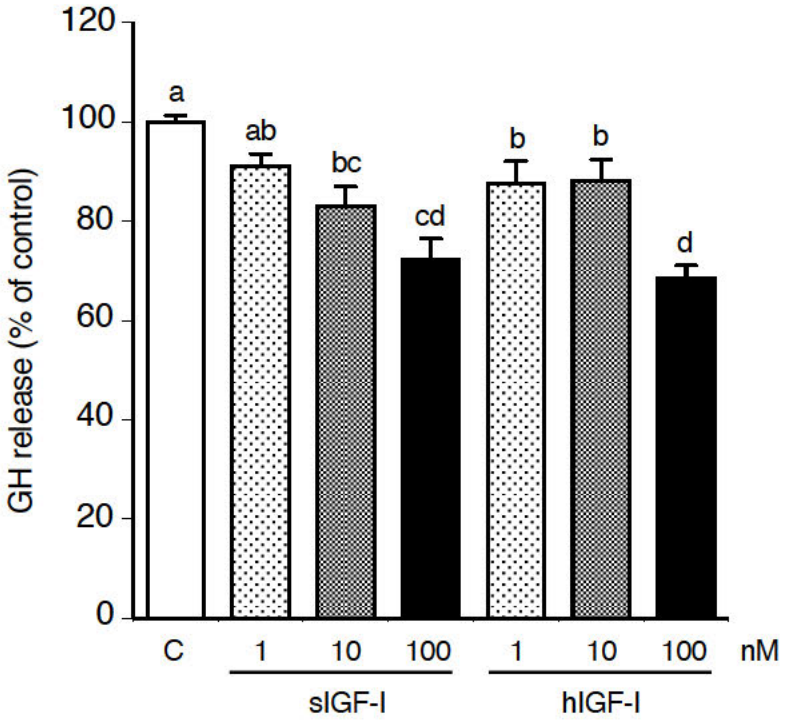
Hasegawa et al., Fig. 5



Hasegawa et al., Fig. 6



Hasegawa et al., Fig. 7



Hasegawa et al., Fig. 8

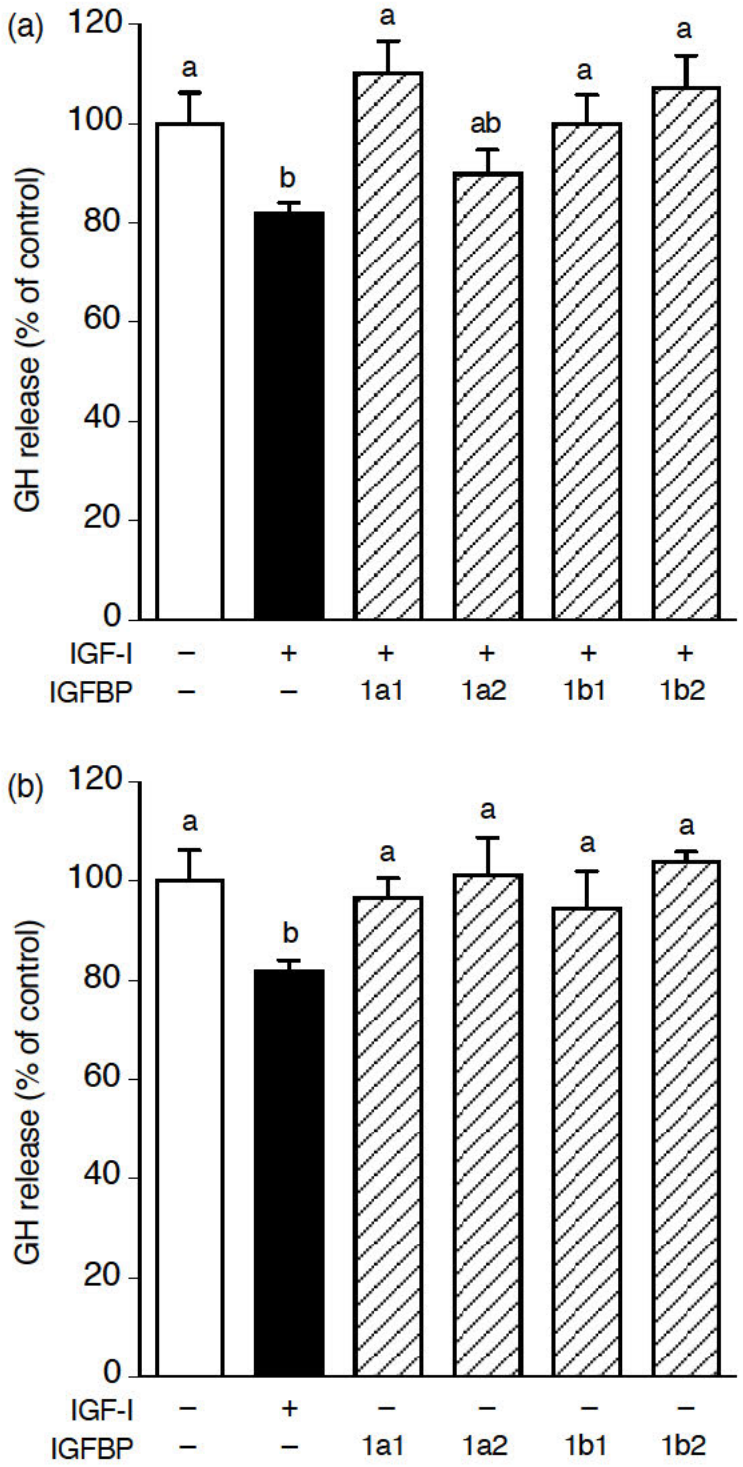


Table 1 Primer sequences used for cDNA cloning and subcloning

Primer name	Primer sequence (5'-3')	Direction	Use
IGFBP-1a2 F	CTACCAGACTCTCCGTTTACA	Forward	RT PCR
IGFBP-1a2 R	CTGCATCAGTGGTTGATCTC	Reverse	RT PCR
IGFBP-1b1 F	ATGCTTGGATTATATGAGAAGTTGACC	Forward	RT PCR
IGFBP-1b1 R	CCTGTGAATGTGGGTCAGAGTG	Reverse	RT PCR
pET32a F	GCCATGGCTTGATATCGGATC	Forward	Inverse PCR
pET32a R	CTTGTCGTCGTCGTCGGTAC	Reverse	Inverse PCR
pET32a-IGFBP-1a2 F	GACGACGACGACAAGTCCCCGGTGTTAGCTCAAGA	Forward	Inverse PCR
pET32a-IGFBP-1a2 R	GATATCAGCCATGGCTTAAGAACATTCTGCATCTCCAGAC	Reverse	Inverse PCR
pET32a-IGFBP-1b1 F	GACGACGACGACAAGTCCCCTGTGGTGGGCCCGGAGCCTA	Forward	Inverse PCR
pET32a-IGFBP-1b1 R	GATATCAGCCATGGCTTAGAGCTCCAGCTGGCACTCTAAGCCG	Reverse	Inverse PCR

Underlines are sequences complement to pET-32a

Table 2 Amino acid sequence homologies among masu salmon IGFBP-1 subtypes.

	IGFBP-1a1	IGFBP-1a2	IGFBP-1b1	IGFBP-1b2
IGFBP-1a1		77%	63%	63%
IGFBP-1a2	77%		56%	56%
IGFBP-1b1	63%	56%		82%
IGFBP-1b2	63%	56%	82%	

Table 3 Recovery of IGFBP-1a2 and -1b1 during purification steps.

	IGFBP-1a2 (μg)	IGFBP-1b1 (μg)
Refolded sample	602	441
Digested sample	386	427
Purified sample	26	24
Recovery (%)	4.3	5.5