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Production of recombinant masu salmon insulin-like growth factor binding protein-2b1 and its action on pituitary cells

Authors:

Takuto Miura[1], Sota Watanabe[2], Ryosuke Kizaki[3], Ryuya Hasegawa[1], Taiga Isozaki[2], and Munetaka Shimizu[4,5]

Affiliations:

[1]Graduate School of Environmental Science, Hokkaido University, Kita 10, Nishi 5, Kita-ku, Sapporo, Hokkaido 060-0810, Japan

[2]School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido 041-8611, Japan

[3]Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido 041-8611, Japan

[4]Faculty of Fisheries Sciences, , Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido 041-8611, Japan

[5]Field Science Center for Northern Biosphere, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido 041-8611, Japan

*Corresponding author, e-mail: mune@fish.hokudai.ac.jp; office: +81-138-40-8897

Abstract

Insulin-like growth factor binding protein (Igfbp)-2b is believed to be a major carrier of circulating Igf-1 in salmonids. We cloned cDNAs of two paralogs of *igfbp-2b* from the liver of masu salmon and produced recombinant Igfbp-2b1 corresponding to the circulating form using a bacterial expression system. The deduced amino acid sequence of masu salmon *igfbp-2b1* had a 75.2% sequence identity with that of masu salmon *igfbp-2b2*, and 88.7% and 96.5% with those of Atlantic salmon and rainbow trout *igfbp-2b1*, respectively. The coding region of masu salmon *igfbp-2b1* cDNA was subcloned into the pET-16b or pET-32a vector and expressed using either a histidine (His)-tag or a thioredoxin (Trx) and His-tag. Recombinant masu salmon (rs) Igfbp-2b1 with the fusion partner was fractionated in the precipitate, solubilized, and isolated using Ni-affinity chromatography. His.rsIgfbp-2b1 and Trx.His.rsIgfbp-2b1 were treated with Factor Xa and enterokinase K, respectively, to remove the fusion partner; only the digestion with enterokinase was successful. After enzymatic digestion, rsIgfbp-2b1 was purified employing reversed-phase high-performance liquid chromatography. The purified rsIgfbp-2b1 was added to a primary culture of masu salmon pituitary cells with or without human (h) IGF-1 to assess its effect on the release of growth hormone (Gh). Although addition of hIGF-1 alone had no effect on Gh release, co-incubation with varying amounts of rsIgfbp-2b1 increased Gh release in a dose-dependent manner. In addition, rsIgfbp-2b1 in the absence of hIGF-1 showed a positive effect on Gh release from salmon pituitary cells. These results suggest that rsIgfbp-2b1 may either have Igf-1–independent action on Gh release or inhibits the suppressive effect of local pituitary Igf-1 on Gh release.

Keywords

Growth regulation; Insulin-like growth factor; Recombinant protein; Salmon

1. Introduction

The growth hormone (GH)-insulin-like growth factor (IGF)-1 system is central to the regulation of postnatal growth in vertebrates. Although IGF-1 is expressed in virtually all tissues and exerts its some actions in an autocrine and or paracrine manner (LeRoith et al., 2001, 2021), it is mainly produced by the liver in response to stimulation by GH (Daughaday and Rotewein, 1989). Hepatic IGF-1 is secreted into the bloodstream where it mediates the action of GH. Circulating IGF-1 generally suppresses GH release from the pituitary cells and forms a negative feedback loop to balance growth (Ohlsson et al., 2009).

Regardless of the mode of action, availability of IGF-1 to its receptor is tightly regulated by high-affinity IGF-binding proteins (IGFBPs). Six IGFBPs have been identified in mammals (Shimasaki and Ling, 1991; Bach, 2018). IGFBP-2 and -3 are the two most abundant forms present in the circulation (Rajaram et al., 1997; Baxter, 2023). IGFBP-2 has a core molecular weight of 31 kDa and displays no major post-translational modifications, such as glycosylation and phosphorylation (Russo et al., 2015; Bach, 2018). IGFBP-2 generally inhibits the IGF-1 action by preventing it from interacting with its receptor. In contrast, IGFBP-3 is N-glycosylated and appears as a doublet band at 43-45 kDa on the gel (Martin and Baxter, 1992). IGFBP-3 carries approximately 75-80% of circulating IGF-1 by forming a ternary complex of 150 kDa with IGF-1 and an acid-labile subunit (ALS), which prevents IGF-1 from being filtered by the glomeruli in the kidneys (Baxter and Martin, 1989; Zapf, 1995; Rajaram et al., 1997).

Genes encoding the six IGFBPs are conserved in vertebrates, including fishes (Wood et al., 2005a; Ocampo Daza et al., 2011). Due to an extra round of whole-genome duplication, teleosts generally possess two subtypes of the six IGFBPs, except for IGFBP-4 (Ocampo Daza et al., 2011). Moreover, autotetraploidization in salmonids generate two paralogs of each subtype. Salmonids have four genes that code for *igfbp-1* (i.e., 1a1, 1a2, 1b1, and 1b2). Thus, 22 genes encoding *igfbp*s have been identified in Altantic salmon (*Salmon salar*) and rainbow trout (*Oncorhynchus mykiss*) (Macqueen et al., 2013; de la Serrana and Macqueen, 2018). The fact that these duplicated genes are conserved in salmonids suggests that the fine-tuning of Igf action by Igfbps has a functional advantage (Allard et al., 2018). Therefore, unraveling the function of each Igfbp paralog is important.

In salmonid circulation, three to four Igfbps have been detected by ligand blotting using labeled human IGF-1 (Niu and LaBail, 1993; Shimizu et al., 1999; Kelley et al., 2001; Shimizu and Dickhoff, 2017). One of these Igfbps has a molecular weight similar to that of human IGFBP-3 (i.e. 41-43 kDa). In addition, the 41-43 kDa Igfbp is high under feeding conditions and is induced in the blood by administration of GH. Based on its molecular weight and physiological regulation, it has been postulated to be a physiologic Igfbp-3 in salmonids (Shimizu et al., 2003a,b). However, the amino acid sequence of purified and cloned salmon 41-43 kDa Igfbp was

most closely related to that of IGFBP-2 among the six human IGFBPs and was classified as Igfbp-2b (Shimizu et al., 2011). In contrast, none of the circulating Igfbps in salmonids corresponded to Igfbp-3 (Shimizu and Dickhoff, 2017). In addition, there is no evidence of the presence of Igfbp-3 in the circulation of salmonids and other fish species, while *als* is expressed in the liver of salmonids (Shimizu et al., 2011; Shimizu and Dickhoff, 2017) and has recently been found to vary in gene expression in other fishes in patterns associated with nutritional intake (Bersin et al., 2023) or individual growth rate (Haldar et al., 2022). These findings suggest that salmonid Igfbp-2b and human IGFBP-3 function in a similar way to promote growth by protecting Igf-1/IGF-1 and enhancing its availability to receptors present on target tissues (Shimizu and Dickhoff, 2017). However, this hypothesis has not yet been proven.

We recently produced a rainbow trout line with the *igfbp-2b* genes disrupted using the CRISPR/Cas 9 system (Cleveland et al., 2018, 2020). Although circulating Igfbp-2b was barely detected after the disruption of functional *igfbp-2b* genes, effects on circulating Igf-1 levels and growth were modest, likely due to compensatory responses of the whole organism (Cleveland et al., 2020). These findings imply that a combination of *in vivo*/*in vitro* and loss-of-function/gain-of-function analyses is necessary, as is the case in zebrafish models (Duan et al., 1999; Wood et al., 2005b; Zhou et al., 2008). The production of recombinant proteins is desirable for this approach. The present study reports the production of recombinant salmon Igfbp-2b and examines its effect on Gh release from cultured pituitary cells of masu salmon.

2. Materials and Methods

2.1. Cloning of partial cDNAs encoding mature proteins of masu salmon igfbp-2b1 and -2b2

A cDNA template prepared from the liver of wild masu salmon smolts (Hasegawa et al., 2020) was used for cDNA cloning. Primers were designed based on the cDNA sequences of Chinook salmon *igfbp-2b* (GenBank Accession No. HM358881; Shimizu et al., 2011b) and rainbow trout *igfbp-2b2* (GenBank Accession No. HM358880) to amplify complete open reading frames (ORFs) of masu salmon *igfbp-2b* paralogs (Table 1). Reverse transcriptase (RT)-PCR was performed using AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Foster City, CA) and Veriti Thermal Cycler (Applied Biosystems). PCRs consisted of 1 cycle at 95 °C for 10 min; 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and 1 cycle at 72 °C for 7 min. The PCR products were run on a 1.0% agarose gel, purified, and cloned into the pGEM-T Easy Vector (pGEM-T Easy Vector Systems, Promega, Madison, WI). Positive clones were sequenced as described by Shimizu et al. (2011).

2.2. Construction of expression vectors and expression of recombinant proteins

pET-16b and pET-32a(+) vectors (Novagen, Madison, WI, USA), which had a His-tag (6 × His)

or a thioredoxin (Trx) and His-tag, respectively, added to the N-terminal region of the expressed recombinant protein were used as expression vectors. cDNAs encoding the ORFs of *igfbp-2b1* and *-2b2* were subcloned into the expression vectors using the In-Fusion™ Advantage PCR Cloning Kit (Clontech, Takara Bio, Shiga, Japan) according to the manufacturer's instructions; the primers used are listed in Table 1. Detailed procedures for the vector construction and protein expression are described by Tanaka et al. (2018). The constructed plasmids (pET16b-His.rsIgfbp-2b1 and pET32a-Trx.His.rsIgfbp-2b1) were transformed into the *Escherichia coli* strain Rosetta-gami B(DE3)pLysS (Novagen). Expression of His.rsIgfbp-2b1 and Trx.His.rsIgfbp-2b1 was induced by adding isopropyl-β-D-thiogalactoside (IPTG). Bacterial proteins were separated into soluble and insoluble fractions using the Bugbuster Protein Extraction Reagent (Novagen). The insoluble fractions containing the recombinant proteins were solubilized in 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 in a stepwise manner to facilitate correct refolding of the proteins.

2.3. Enzymatic digestion and purification of recombinant proteins

After dialysis, the His tag was cleaved from His.rsIgfbp-2b1 by Factor Xa using Restriction Grade Factor Xa Kit (Novagen). In brief, 10 μg of protein was mixed with 0.1, 0.5, or 1.0 unit Factor Xa and incubated for 16 h. Furthermore, the fusion partner (i.e., Trx.His) of Trx.His.rsIgfbp-2b1 was cleaved by an enterokinase using a Tag.off™ High Activity rEK Kit (Novagen). One hundred micrograms of protein was mixed with 1 unit enterokinase and incubated at 20 °C for 30 h. rsIgfbp-2b1 thus obtained was 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 by Tanaka et al. (2018).

2.4. Electrophoresis and ligand blotting

The recombinant proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 3% stacking gel and 12.5% separating gel. In brief, the samples were mixed with an equal volume of a sample buffer containing 2% SDS and 10% glycerol with or without 5% 2-mercaptoethanol at 100 °C for 2 min. Next, the samples 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 using the Precision Marker (Bio-Rad).

IGF-binding ability of the recombinant proteins was assessed by ligand blotting with digoxigenin-labeled human IGF-1 (DIG-hIGF-1) as described by Shimizu et al. (2000). In brief, after electrophoresis, the proteins were electroblotted onto a nitrocellulose membrane. The

membrane was incubated with 50 ng/mL DIG-hIGF-1 for 2 h at room temperature (25 °C) and then with an antibody against DIG conjugated with horseradish peroxidase (Roche, Indianapolis, IN, USA) 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, USA) and ImageQuant LAS 500 (GE Healthcare, Uppsala, Sweden).

2.5. Primary pituitary cell culture

Primary pituitary cell culture was carried out according to the methods of Ando et al. (2004) with modifications as described by Tanaka et al. (2018). For an initial experiment, pituitaries were collected from 70 immature (one-year-old) masu salmon of both sexes (fork length 22.8 ± 0.5 cm, body weight 121.5 ± 9.0 g; expressed as mean ± SE of 10 fish) at the Toya Lake Station, Field Science Center for Northern Biosphere, Hokkaido University (Abuta-gun, Hokkaido, Japan) in November 2020. The sampling was performed in accordance with the guidelines of the Hokkaido University Animal Care and Use Committee (Approval No. 30-3). The pituitaries were pooled, minced and digested with 0.1% collagenase at 12 °C for 20 h. The dispersed cells were plated at a density of 1.75×10^5 cells/1.3 mL medium/well (n = 3–5) on 12-well culture plates (BD Biosciences, Franklin Lakes, NJ) pre-coated with 50 μg/mL poly-D-lysine hydrobromide (Sigma, St. Louis, MO, USA), and incubated in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with Serum Replacement 2 (Sigma-Aldrich, Tokyo, Japan) at 12 °C for 48 h. rsIgfbp-2b1 at a final concentration of 25, 100, or 400 nM with or without 100 nM hIGF-1(GroPep Bioreagents, Thebarton, Australia) was added to the wells, while only the medium was added to the control wells. The cells were incubated for additional 24 h and culture medium from each well was collected at 12 and 24 h.

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

Gh levels in the medium were quantified using TR-FIA. A competitive method was employed to perform the DELFIA immunoassay (PerkinElmer, Waltham, MA, USA) according to the manufacturer's instructions. Purified chum salmon Gh and Gh labeled with europium (Eu-sGh) using a DELFIA Eu-N1 ITC chelate labeling kit (PerkinElmer) were provided by Shunsuke Moriyama and Arimune Munakata, respectively. Antiserum against purified chum salmon Gh that had been used for radioimmunoassays (Björnsson et al., 1994) was provided by Akihiko Hara (Hokkaido University, Japan). 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 (1:40,000) in a 96-well microtiter plate coated with goat anti-rabbit IgG (PerkinElmer) overnight at 4 °C. Next, 40 μl Eu-Gh (1.4 ng) was added to each well, and the plate was incubated overnight at 4 °C. After washing with DELFIA Wash Buffer (PerkinElmer), 200 μL DELFIA

Enhancement Solution (PerkinElmer) was added to each well. Time-resolved fluorescence was measured using the Wallac ARVO X4 multilabel counter (Perkin Elmer).

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, USA). When significant effects were found, the differences were further verified using Tukey's honest significant difference (HSD) 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-2b1 and -2b2

Deduced amino acid (aa) sequences of the cloned masu salmon *igfbp-2b1* (GenBank Accession No. PP860492) and *igfbp-2b2* (GenBank Accession No. PP860493) consisted of 283 aa (29.1 kDa) and 286 aa (29.7 kDa), respectively (Fig. 1). They shared a 75.2% sequence homology. Amino acid sequence homologies of masu salmon Igfbp-2b1 with Atlantic salmon and rainbow trout Igfbp-2b1 were 88.7 and 96.5%, respectively; the corresponding paralogs of masu salmon Igfbp-2b2 were 91.6 and 94.1%, respectively, Masu salmon Igfbp-2b1 contained 18 cysteine residues that are conserved in the IGFBP family at the N- and C-termini, while masu salmon Igfbp-2b2 lacked the sixth cysteine residue located at the N-terminus but had an extra cysteine after the ninth cysteine (Fig. 1). Masu salmon Igfbp-2b1 had three potential N-glycosylation sites (Asn-X-Ser/Thr) that are not present in the human ortholog but are conserved among salmonid counterparts. In contrast, masu salmon Igfbp-2b2 had one potential N-glycosylation site that was also found in the rainbow trout ortholog (Fig. 1) but not in Atlantic salmon ortholog (data not shown). An integrin-binding site, Gly-Arg-Asp (RGD), was observed to be conserved in both masu salmon Igfbp-2b1 and -2b2 (Fig. 1).

3.2. Expression of recombinant masu salmon Igfbp-2b1

The recombinant masu salmon Igfbp-2b1 protein was expressed using a His-tag. Addition of IPTG produced a band at 34 kDa when SDS-PAGE was performed under reducing conditions; it was present exclusively in the insoluble fraction (Fig. 2a). After solubilization, this protein was isolated using Ni-affinity chromatography and refolded using dialysis. Purified His.rsIgfbp-2b1 exhibited a band at 34 kDa on SDS-PAGE under non-reducing conditions (Fig. 2b). When His.rtIgfbp-2b1 was treated with increasing amounts of Factor Xa, the band observed at 34 kDa that exhibited IGF-binding ability on ligand blotting disappeared at the concentration of Factor Xa > 0.5 unit (Fig. 2c).

We next expressed rsIgfbp-2b1 using Trx and His tags. An IPTG-induced band at 47

kDa was found in both soluble and insoluble fractions (Fig. 3a); the insoluble fraction was purified using an Ni-affinity column. When purified Trs.His.rsIgfbp-2b1 at 47 kDa on SDS-PAGE under non-reducing conditions was treated with 0.3 unit of enterokinase K, a 29 kDa band that had the ability to bind IGF was observed (Fig. 3b,c). The 29 kDa protein was further purified by reverse-phase HPLC (Fig. 4). The purified protein appeared as two bands at 45 and 29 kDa; both the bands exhibited IGF-binding ability upon ligand blotting (Fig. 5).

3.3. Effects of rsIgfbp-2b1 on Gh release from cultured pituitary cells

Cultured pituitary cells from masu salmon yearlings were incubated with hIGF-1 and/or rs Igfbp-2b1. hIGF-1 had no effect on Gh release from pituitary cells at 12 and 24 h after incubation (Fig. 6). In contrast, co-incubation with hIGF-1 and rsIgfbp-2b1 increased Gh release in a dose-dependent manner. Moreover, the addition of Igfbp-2b1 alone resulted in higher Gh release than that of control non-treated cells 12 h after incubation.

4. Discussion

In this study, we cloned cDNAs for two paralogs of *igfbp-2b* from masu salmon. Deduced amino acid sequences of masu *igfbp-2b1* and *-2b2* showed high sequence homology with their corresponding paralogs in Atlantic salmon and rainbow trout. Masu salmon Igfbp-2b1 and -2b2 also share a high sequence homology and have a Gly-Arg-Asp (RGD) sequence at the C-terminus, similar to other fish and mammalian Igfbp-2/IGFBP-2 (Duan et al., 1999; Zhou et al., 2008). RGD is a motif that allows binding to integrin on the cell surface (Jones et al., 1993), suggesting that masu salmon Igfbp-2bs can adhere to the cell surface. Although the overall amino acid sequences of masu Igfbp-2b1 and -2b2 are similar to each other, their protein structures differ in the number and positions of cysteine residues and potential N-glycosylation.

Masu salmon Igfbp-2b2 is unique in terms of mutations that affect the positions of cysteine residues. Out of 18 cysteine residues, 12 located at the N-terminus and 6 at the C-terminus are well conserved in the IGFBP family and are required for high-affinity binding (Forbes et al., 2012; Bach, 2018). In masu salmon Igfbp-2b1, these cysteine residues are located at the same positions. In contrast, although masu salmon Igfbp-2b2 had 18 cysteine residues, the positions of two cysteine residues at the N-terminus were different. A BLAST search for Igfbp-2b2 of other salmonid species suggests that substitution of cysteine residues is unique to masu salmon Igfbp-2b2 (data not shown). Whether such a positional mutation of the cysteine residues affects IGF binding is not addressed in the present study. However, our preliminary experiment related to the expression of recombinant masu salmon Igfbp-2b2 failed to demonstrate its IGF-binding ability, suggesting that the position of the cysteine residue is important (data not shown). Masu salmon Igfbp-2b1 has an extra cysteine residue in the mid/linker domain, although its

contribution to IGF binding is unknown.

Masu salmon Igfbp-2b1 possesses three potential N-glycosylation sites, similar to Chinook salmon Igfbp-2b1 (Shimizu et al., 2011). In Chinook salmon, a circulating 41-kDa Igfbp has been identified as Igfbp-2b based on partial N-terminal amino acid sequence of the purified protein and deduced amino acid sequence of its cDNA (Shimizu et al., 2011). This Chinook salmon Igfbp-2b is classified as Igfbp-2b1, based on its structural relationship with other salmonid paralogs. At least one of the three N-glycosylation sites found in Igfbp-2b1 appears to be functional because the purified Chinook salmon 41-kDa Igfbp is indeed N-glycosylated (Shimizu et al., 2003b). In contrast, no potential N-glycosylation sites were found in salmon or trout Igfbp-2b2. It is worth noting that in mammals N-glycosylation is observed in IGFBP-3 but not in IGFBP-2 (Firth and Baxer, 2002; Forbes et al., 2012). The presence of carbohydrates in native human IGFBP-3 and salmon Igfbp-2b1 makes their molecular weights larger than other Igfbps and similar each other (Shimizu and Dickhoff, 2017).

Given that Igfbp-2b1 corresponds to the circulating 41-kDa Igfbp, we constructed recombinant proteins for this paralog. In the present study, we used a bacterial expression system to construct recombinant salmon Igfbp-1s (Tanaka et al., 2018; Hasegawa et al., 2020). The difference from other studies was that we used two different expression vectors: pET-16b and pET-32a. The former expresses a target protein with only a His-tag and the latter with a Trx and His-tag. These two expression vectors also differ in the enzyme recognition site for cleaving the fusion partner from the target protein; pET-16b and pET-32a use Factor Xa and enterokinase K, respectively. When His.rsIgfbp-2b1 was treated with Factor Xa, it was degraded despite the lack of a recognition site (i.e., Ile-Glu-Gly-Arg). In contrast, cleavage of the fusion partner from Trx.His.rsIgfbp-2b1 with enterokinase K was acceptable, although the recovery was relatively low. Such nonspecific enzymatic degradation has also been observed in rsIgfbp-1b (Tanaka et al., 2018) despite the lack of a cleavage site (i.e., Asp-Asp-Asp-Asp-Lys). This efficiency needs to be improved by changing the enzyme recognition sites in future studies.

Recombinant salmon Igfbp-2b1 was expressed without glycosylation in an *E. coli* expression system. Firth and Baxter (1999) reported that N-glycosylation of IGFBP-3 had no effect on IGF binding. They also found that glycosylation affected cell surface interaction of recombinant IGFBP-3, which was higher in its non-glycosylated form (Firth et al., 1999). Although we were unable to compare the IGF-binding affinity or potential cell surface interaction between glycosylated and non-glycosylated Igfbp-2b1, the expressed rsIgfbp-2b1 exhibited IGF-binding ability upon ligand blotting. This finding suggests that N-glycosylation is not essential for IGF-binding by Igfbp-2b1.

Purified rs Igfbp-2b1 appeared as two bands at 29 kDa and 45 kDa on the ligand blot. Although the molecular weight of the latter band was similar to that of undigested

Trx.His.rsIgfbp-2b1 (47 kDa), it was copurified with rsIgfbp-2b1 when HPLC was performed, and its position of elution was clearly separated from that of Trx.His.rsIgfbp-2b1. Although its identity is not known at present, it might be a dimer/aggregate of purified rsIgfbp-2b1 or a misfolded rsIgfbp-2b1 retaining IGF-binding ability.

Suppression of GH release from the pituitary gland by circulating IGF-1 is an important mechanism that regulates growth (Ohlsson et al., 2009). Given that most circulating IGF-1 is bound to one of the six IGFBPs, inhibition or stimulation of IGF-1 activity by IGFBP is essential to modulate growth (Rajaram et al., 1997; LeRoith et al., 2021; Baxter, 2023). Thus, a pituitary cell culture system with GH measurement is a useful model to assess function of a particular IGFBP *in vitro*. Indeed, Fruchtman et al. (2002) suggested using excised pituitary glands of hybrid striped bass (*Morone saxatili*s x I) that Igfbps play a significant role in modulating the action of IGF-1 on Gh secretion. In the present study, we first optimized TR-FIA to measure Gh levels in culture media. We had previously used biotin-labeled Gh and avidin-conjugated Eu in TR-FIA (Hasegawa et al., 2020). However, the presence of biotin in culture medium resulted in high baseline values. In the present study, Gh was labeled with Eu to obtain a low baseline value. However, the displacement curves of serial dilutions of the Gh standard and the culture medium did not appear to be parallel at high and low concentrations (data not shown). We calculated an average value of Gh concentrations at different dilutions in a single culture medium and used it as a "standard" to express Gh levels in other culture media.

Negative feedback effect of IGF-1 on Gh release has been widely observed in fishes as well as mammals (Pérez-Sánchez et al., 1992; Blaise et al., 1995; Fruchtman et al., 2000; Wong et al., 2006). In the present study, a high dose of hIGF-1 (100 nM), as compared to circulating concentrations in masu salmon (approximately 10 nM; Kaneko et al., 2020), was used to ensure the effect of heterologous hIGF-1. However, in the present study, hIGF-1 alone had no effect on Gh release from masu salmon pituitary cells. This may have occurred due to seasonal effects or/and life-history stages. Supporting this fact, the effect of hIGF-1 on Gh release from the masu salmon pituitary was either positive or negative (Tanaka et al., 2018; Hasegawa et al., 2020).

The results of the pituitary cell culture were unexpected; however, they demonstrated the biological activity of rsIgfbp-2b1. Addition of rsIgfbp-2b1 increased Gh release in a dose-dependent manner. This effect was observed in the absence of hIGF-1. It is possible that rsIgfbp-2b1 concentrations at 100 and 400 nM might be rather pharmacological than physiological because circulating Igfbp-2b concentrations rarely exceed 20 nM (Izutsu e al., 2023). However, these doses were chosen to make rsIgfbp-2b1 excess to the hIGF-1 added to culture media (i.e., 100 nM). Although reproducibility of the result needs to be confirmed using lower doses of hIGF-1 and rsIgfbp-2b1 in future study, there are several possible explanations for this finding. First, rsIgfbp-2b1 exerted a positive effect on Gh release by inhibiting the activity of endogenous

pituitary Igf-1 and exogenous hIGF-1. Synthesis of Igf-1 by pituitary cells has been reported in teleosts (Eppler et al., 2007). If local endogenous Igf-1 in the pituitary of masu salmon is sufficiently high to suppress basal Gh release, the lack of an effect of exogenous hIGF-1 may be explained. In such situations, the addition of rsIgfbp-2b1 may alleviate the suppressive effects of endogenous Igf-1. Another possibility is that rsIgfbp-2b1 may be able to stimulate Gh production and release through an Igf-independent action, as reported for human IGFBP-2, by binding to integrin via the RGD motif (Schütt et al., 2004) or translocating to the nucleus via nuclear localization signals (i.e., $_{179}$PKKLRPP$_{185}$) (Azar et al., 2014). However masu salmon Igfbp-2b1 had no such classical nuclear localization signal in its sequence. Future studies using site-directed mutagenesis of rsIgfbp-2b1 may address its mode of action.

The procedures employed to produce recombinant proteins in this study have important applications to establish immunoassays and *in vivo* functional studies. Immunoassays using purified Chinook salmon Igfbps have been described previously (Shimizu et al., 2003a, 2006). However, purifying Igfbps from salmon serum is labor-intensive. We recently established a TR-FIA using recombinant rainbow trout (rt) Igfbp-2b1 with a Trx and His-tag to prepare an Eu-label (Izutsu et al., 2023). In addition, because Trx.His.rsIgfbp-2b1 had ability to bind IGF-1, the production of large amounts of Trx.His.rsIgfbp-2b1 may be useful for injecting fish to carry out *in vivo* studies. More specifically, producing Trx.His.rtIgfbp-2b1 and injecting it into a rainbow trout line that lacks functional Igfbp-2b via gene editing provides an opportunity to conduct loss-of-function/gain-of-function analysis in fish.

In summary, we produced nonglycosylated rsIgfbp-2b1 without a fusion partner. rsIgfbp-2b1 appeared to be functional because it had IGF-binding ability and an effect on Gh release from the pituitary gland. However, its mode of action is currently unknown and warrants further studies.

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Figure legends

Fig. 1. Comparison of deduced amino acid sequences of cloned masu salmon *igfbp-2b*s with those of human and rainbow trout *igfbp-2b*s. Amino acid sequences of human *IGFBP2* (NP_000588), and rainbow trout *igfbp-2b1* (NM_001124557.1) and *-2b2* (JX674936) were obtained from GenBank. They were aligned by the ClustalW method. Cysteine residues conserved in the IGFBP family and those not conserved are asterisked and circled, respectively. Potential N-glycosylation sites are depicted in boxes with solid lines. The position of Arg-Gly-Asp (RGD) integrin recognition sequence is underlined with solid lines. A classical nuclear localization signal is underlined with double-dashed lines.

Fig. 2. (a) SDS-PAGE of extracts of bacterial cells transformed with pET16b-His.Igfbp-2b1 with (+) and without (-) addition of isopropyl-β-D-thiogalactoside (IPTG) to the culture medium. Expressed bacterial protein after addition of IPTG were fractionated into soluble and insoluble fractions and separated on 12.5% gels under reducing conditions. Migration positions of the molecular weight marker are indicated by arrowheads on the left. Arrowhead on the right indicate induced recombinant fusion protein along with its molecular weight. (b) Enzymatic cleavage of His.rsIgfbp-2b1. Ten micrograms of recombinant proteins were digested with 0, 0.1, 0.5, or 1.0 unit of Factor Xa at room temperature (25 ℃) for 16 h, subjected to 12.5% SDS-PAGE under non-reducing conditions, and stained with Coomassie Brilliant Blue (CBB; left panel). The same samples were electroblotted onto a nitrocellulose membrane following SDS-PAGE and subjected to ligand blotting (LB; right panel) using digoxigenin-labeled human IGF-1 (50 ng/ml) and antiserum against digoxigenin (1:10,000). Arrowhead indicates the migration position and molecular weight of undigested His.rsIgfbp-2b1.

Fig. 3. (a) SDS-PAGE of extracts of bacterial cells transformed with pET32a-Trx.His.rsIgfbp-2b1 with (+) and without (-) addition of isopropyl-β-D-thiogalactoside (IPTG) to the culture medium. Expressed bacterial protein after addition of IPTG were fractionated into soluble and insoluble fractions and separated on 12.5% gels under reducing conditions. Migration positions of the molecular weight marker are indicated by arrowheads on the left. Arrowhead on the right indicate induced recombinant fusion protein along with its molecular weight. (b) Enzymatic cleavage of rsIgfbp-2b1. Thirty micrograms of Trx.His.rsIgfbp-2b1 was incubated with 0.3 unit of enterokinase (EK) at 20 ℃ for 3 h, subjected to 12.5% SDS-PAGE under non-reducing condition, and stained with Coomassie Brilliant Blue (CBB; left panel). The same samples were electroblotted onto a nitrocellulose membrane following SDS-PAGE and subjected to ligand blotting (LB; right panel) using digoxigenin-labeled human IGF-1 (50 ng/ml) and antiserum against digoxigenin (1:2,000). Arrowheads on the right indicate undigested (47kDa) and digested

(29kDa) proteins.

Fig.4. Purification of rsIgfbp-2b1 by reversed-phase HPLC. The digested sample (S) was applied to a Vydac C-4 column and eluted using a linear gradient of 0–100% acetonitrile in 0.1% trifluoroacetic acid (TFA; dashed line). Major peaks were collected and analyzed by ligand blotting (inset). Arrowheads on the ligand blot indicate migration positions of undigested (47 kDa) and digested (29 kDa) rsIgfbp-2b1.

Fig. 5. SDS-PAGE and ligand blotting of purified rsIgfbp-2b1. One-point-three micrograms of purified protein was separated using 12.5% gels under non-reducing conditions (left panel), followed by analysis using ligand blotting (LB; right panel) with digoxigenin-labeled human IGF-1 (50 ng/ml) and antiserum against digoxigenin (1:2,000). Migration positions of the molecular weight markers are indicated by arrowheads on the left.

Fig. 6. Effects of addition of rsIgfbp-2b1 on Gh release from cultured masu salmon pituitary cells. Pooled pituitary cells from one-year-old immature male and female masu salmon were pre-cultured for 2 days and incubated for 12 h (a) and 24 h (b) without or with 100 nM human IGF-1 (hIGF-1) and varying concentrations (nM) of rsIgfbp-2b1 (sBp-2b1). Treatments sharing the same letters are not significantly different from each other. (n = 3–5). Control cells (n = 5) were not treated with either hIGF-1 nor Igfbp-2b1. Symbols sharing the same letters are not significantly different from each other (Tukey's HSD test, $P < 0.05$).

Figure 1

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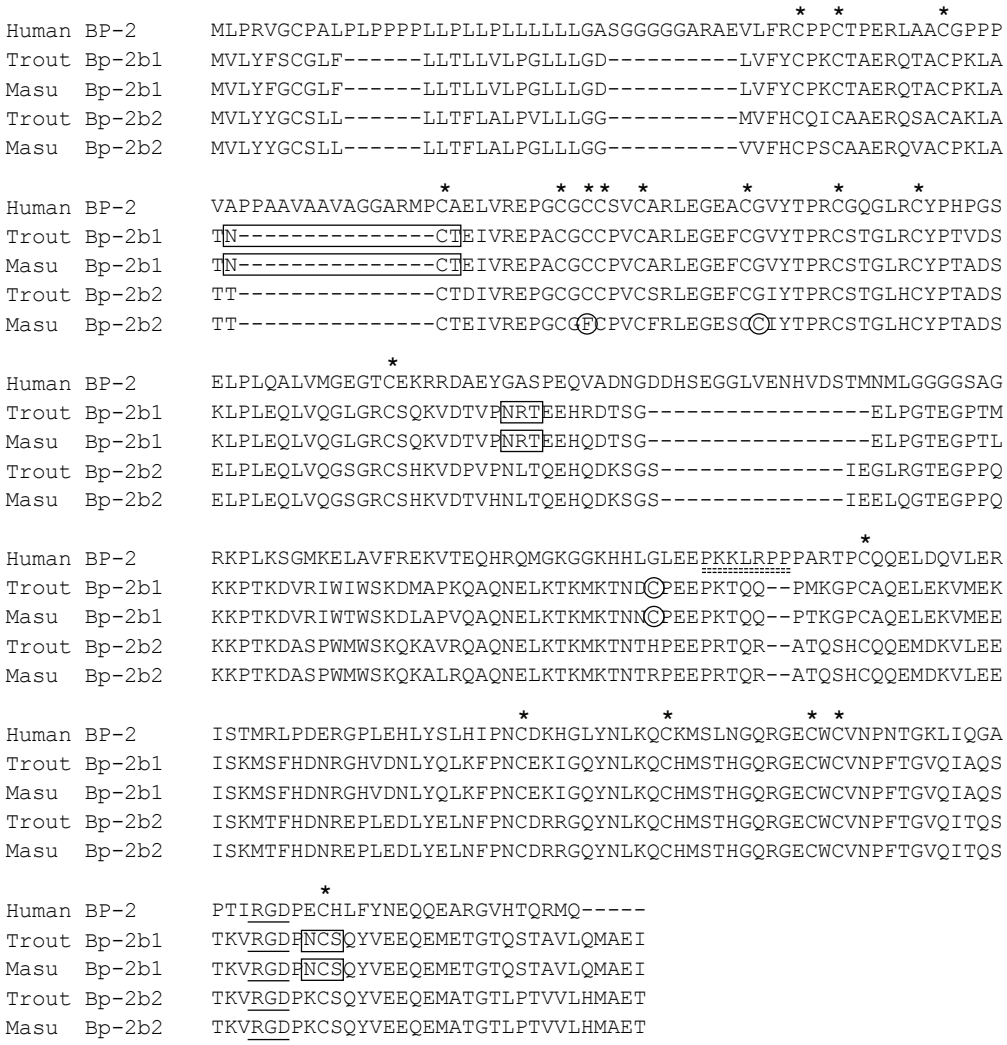
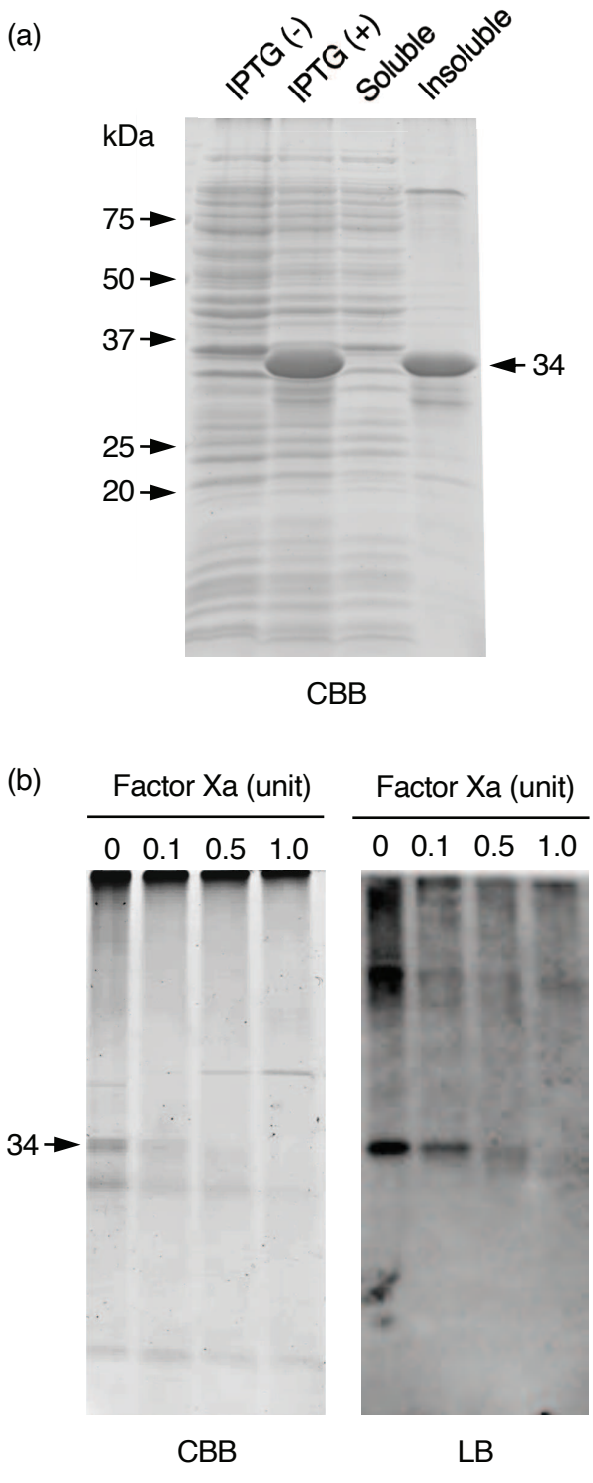
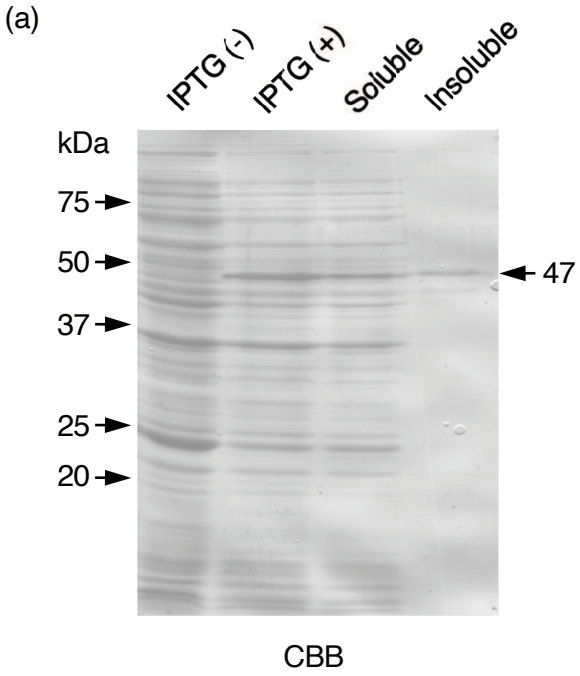
Miura et al., Fig. 1

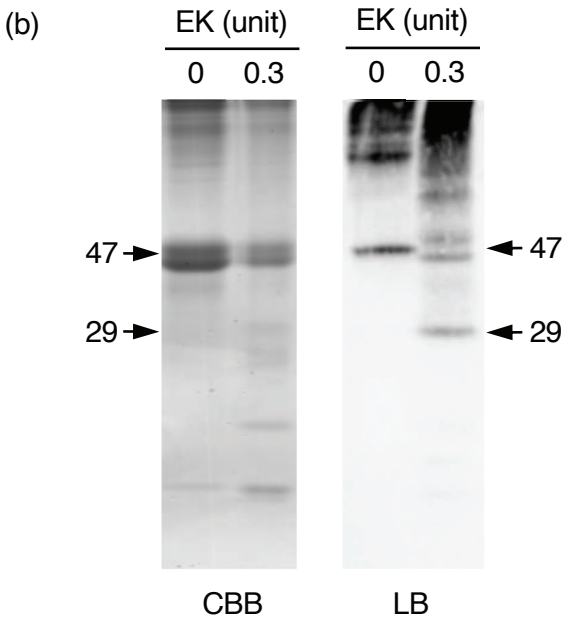


Miura et al., Fig. 2



Miura et al., Fig. 3





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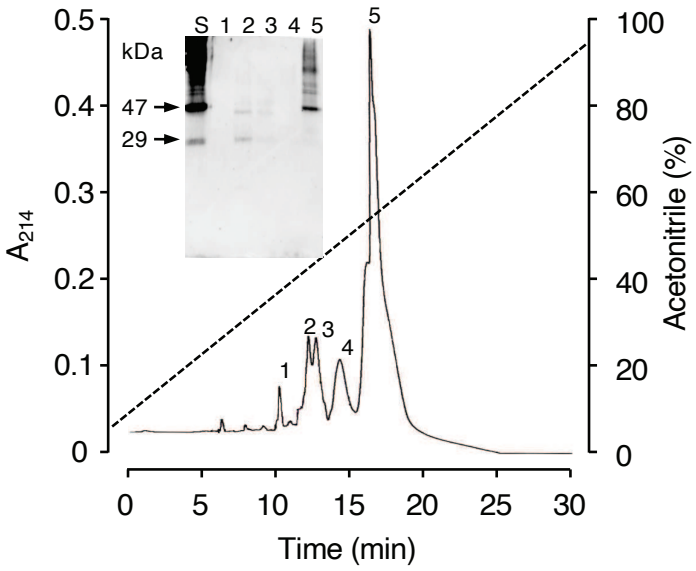

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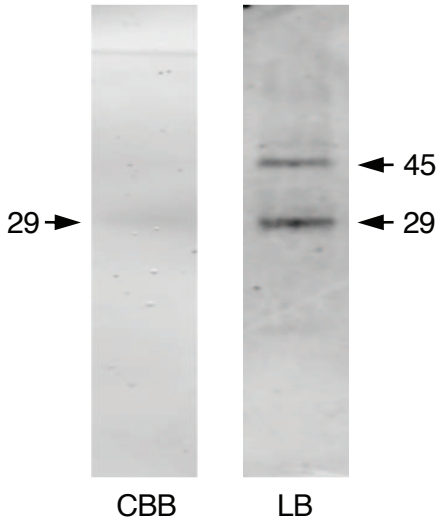

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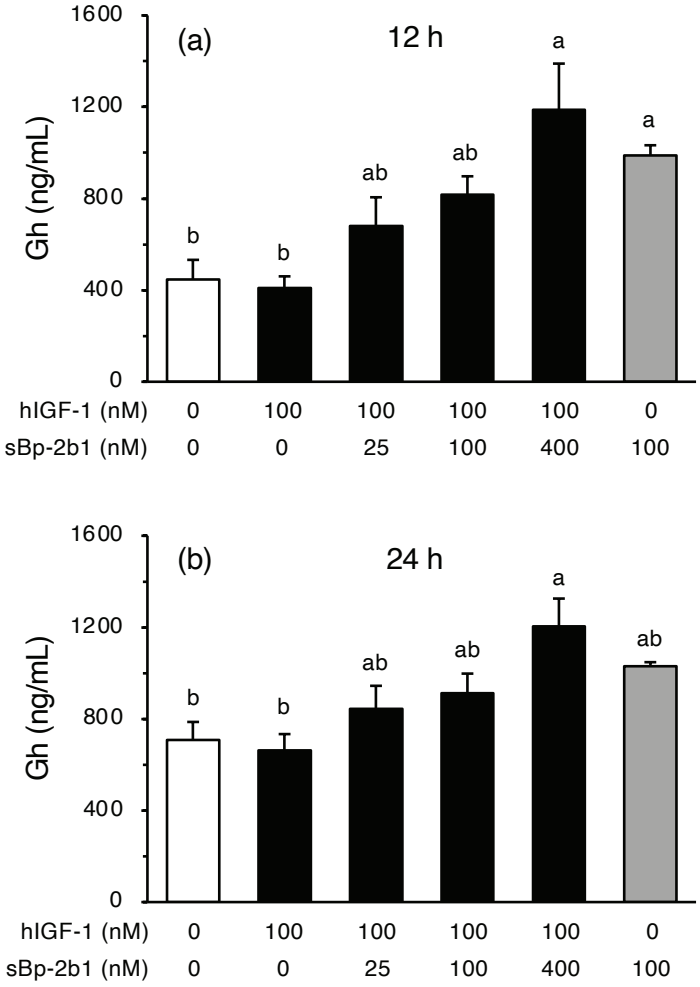

Table 1 Primer sequences used for cDNA cloning and subcloning.

Primer Name	Primer sequence (5'-3')	Direction	Use
igfbp-2b1 F	AATAAACAGTACAGCTAGAGGCAGA	Forward	RT PCR
igfbp-2b1 R	GTATCTGTGTGTGAGTGAAGTGCTG	Reverse	RT PCR
igfbp-2b2 F	CTCTTTATTAGACGGTACAGCTCG	Forward	RT PCR
igfbp-2b2 R	TCTGGATGTGGGTGAAGCTC	Reverse	RT PCR
pET16b F	CATATGCTCGAGGATCCG	Forward	Inverse PCR
pET16b R	ACGACCTTCGATATGGCC	Reverse	Inverse PCR
pET16b-igfbp-2b1 F	CATATCGAAGGTCGTGACTTGGTATTTTATTGTCCGAAATGC	Forward	InFusion PCR
pET16b-igfbp-2b1 R	ATCCTCGAGCATATGTTATATCTCTGCCATCTGCAGGAC	Reverse	InFusion PCR
pET32a F	GCCATGGCTGATATCGGATC	Forward	Inverse PCR
pET32a R	CTTGTCGTCGTCGTCGGTAC	Reverse	Inverse PCR
pET32a-igfbp-2b1 F	GACGACGACGACAAGGACTTGGTATTTTATTGTCCGAAATGC	Forward	InFusion PCR
pET32a-igfbp-2b1 F	GATATCAGCCATGGCTTATATCTCTGCCATCTGCAGGAC	Reverse	InFusion PCR

Underlines are sequences complement to pET-16b or pET-32a.