



Title	Production of recombinant salmon insulin-like growth factor binding protein-1 subtypes
Author(s)	Tanaka, Hanae; Oishi, Gakuto; Nakano, Yusuke; Mizuta, Hiroko; Nagano, Yuta; Hiramatsu, Naoshi; Ando, Hironori; Shimizu, Munetaka
Citation	General and Comparative Endocrinology, 257, 184-191 https://doi.org/10.1016/j.ygcen.2017.06.015
Issue Date	2018-02-01
Doc URL	http://hdl.handle.net/2115/72472
Rights	© 2018. This manuscript version is made available under the CC-BY-NC-ND 4.0 license https://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	article (author version)
File Information	Tanaka18HUSCAP.pdf



[Instructions for use](#)

1 **Title:**

2 Production of recombinant salmon insulin-like growth factor binding protein-1 subtypes

3

4 **Authors:**

5 Hanae Tanaka¹, Gakuto Oishi¹, Yusuke Nakano¹, Hiroko Mizuta¹, Yuta Nagano¹, Naoshi
6 Hiramatsu¹, Hironori Ando² and Munetaka Shimizu^{1*}

7

8 ¹Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido
9 041-8611, Japan

10

11 ²Sado Marine Biological Station, Faculty of Science, Niigata University, 87 Tassha, Sado
12 952-2135, Japan

13

14 *Corresponding author at: Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato,
15 Hakodate, Hokkaido 041-8611, Japan.

16 Fax: +81 138 40 8897. E-mail address: mune@fish.hokudai.ac.jp (M. Shimizu)

17

18 **Abstract**

19 Insulin-like growth factor (IGF)-I is a growth promoting hormone that exerts its actions through
20 endocrine, paracrine and autocrine modes. Local IGF-I is essential for normal growth, whereas
21 circulating IGF-I plays a crucial role in regulating the production and secretion of growth
22 hormone (GH) by the pituitary gland. These actions of IGF-I are modulated by six insulin-like
23 growth factor binding proteins (IGFBPs). In teleosts, two subtypes of each IGFBP are present due
24 to an extra round of whole-genome duplication. IGFBP-1 is generally inhibitory to IGF-I action
25 under catabolic conditions such as fasting and stress. In salmon, IGFBP-1a and -1b are two of
26 three major circulating IGFBPs and assumed to affect growth through modulating IGF-I action.
27 However, exact functions of salmon IGFBP-1 subtypes on growth regulation are not known due
28 to the lack of purified or recombinant protein. We expressed recombinant salmon (rs) IGFBP-1a
29 and -1b with a fusion protein (thioredoxin, Trx) and a His-tag using the pET-32a(+) vector
30 expression system in *Escherichia coli*. Trx.His.rsIGFBP-1s were isolated by Ni-affinity
31 chromatography, enzymatically cleaved by enterokinase to remove the fusion partners and further
32 purified by reversed-phase HPLC. We next examined effects of rsIGFBP-1a and -1b in
33 combination with human IGF-I on GH release from cultured masu salmon (*Oncorhynchus*
34 *masou*) pituitary cells. Unexpectedly, IGF-I increased GH release and an addition of rsIGFBP-1a,
35 but not rsIGFBP-1b, restored GH levels. The results suggest that IGFBP-1a can inhibit IGF-I
36 action on the pituitary in masu salmon. Availability of recombinant salmon IGFBP-1s should
37 facilitate further functional analyses and assay development.

38

39 Keywords: insulin-like growth factor binding protein-1; recombinant protein; salmon; pituitary
40 cell culture; growth hormone release

41

42 **1. Introduction**

43 Insulin-like growth factor (IGF)-I is a 7.5 kDa peptide hormone produced mainly by the liver in
44 response to stimulation by growth hormone (GH) (Daughaday and Rotwein, 1989). Hepatic IGF-I
45 is secreted into the bloodstream and mediates many actions of GH. IGF-I is also expressed in
46 virtually all tissues and regulates cell proliferation, differentiation, growth and apoptosis in
47 paracrine and autocrine modes (Le Roith et al., 2001). Local IGF-I is essential for postnatal
48 growth, whereas endocrine IGF-I is important for regulating circulating GH by inhibiting its
49 synthesis and secretion at the pituitary level, as well as the hypothalamic level (Ohlsson et al.,
50 2009).

51 Although the contribution of endocrine IGF-I in postnatal growth of mammals may
52 not be as significant as local IGF-I, it forms a relatively large pool in the circulation and affects
53 many tissues. This can be achieved by the presence of six IGF-binding proteins (IGFBPs).
54 IGFBPs are not structurally related to the IGF-receptor, but are single chain peptides 23-31 kDa in
55 size, consisting of three domains (Firth and Baxter, 2002; Forbes et al., 2012). The cysteine-rich
56 N- and C-terminal domains are required for high-affinity IGF-binding and the mid linker
57 (L)-domain contains sites for phosphorylation, glycosylation and enzymatic cleavage that are
58 specific to each IGFBP (Firth and Baxter, 2002; Forbes et al., 2012). IGFBPs prolong the half-life
59 of IGF-I from 5-10 min up to 12 hr by forming a high-molecular weight complex which prevents
60 IGF-I from being ultrafiltered by the kidney and protects it from enzymatic degradation (Rajaram
61 et al., 1997). IGFBPs can either inhibit or promote IGF-I action through regulating the availability
62 of IGF-I to its receptor in target tissues. In addition, some IGFBPs translocate into the nucleus and
63 regulate gene transcription independent of IGF-I (Forbes et al., 2012).

64 Multiple whole-genome duplication events in combination with local modifications
65 shaped the number of IGFBP genes. Phylogenetic studies suggest that six vertebrate IGFBPs
66 were created first by a local duplication of an ancestral protein (two genes) followed by two
67 whole-genome duplications (eight genes) and subsequent loss of two genes (six genes) (Daza et
68 al., 2011). Since teleosts experienced an extra round of whole-genome duplication, they usually
69 have two copies of each member of the six IGFBPs, except IGFBP-4 (Daza et al., 2011).
70 Moreover, a recent study by Macqueen et al. (2013) demonstrated that salmonids such as Atlantic
71 salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) have 19 IGFBP subtypes due to
72 their tetraploid origin. These studies highlight the presence of multiple IGFBP subtypes in fish
73 and suggest their functional partitioning.

74 IGFBP-1 is one of the major IGFBPs in the circulation and generally inhibitory to IGF

75 action by preventing it from interacting with its receptor (Lee et al., 1993, 1997; Wheatcroft and
76 Kearney, 2009). Unlike other IGFBPs, IGFBP-1 shows dramatic daily changes in response to
77 meals. Insulin is the major inhibitor of IGFBP-1 production, whereas cortisol stimulates its
78 production (Lee et al., 1993, 1997; Wheatcroft and Kearney, 2009). These findings suggest that
79 IGFBP-1 is important for glucose regulation under catabolic conditions.

80 Fish likely possess two IGFBP-1s in their circulation. In the fish circulation, three
81 IGFBPs are consistently detected around 20-25, 28-32 and 40-50 kDa (Kelley et al., 2001). The
82 two low-molecular-weight IGFBPs were assumed to be IGFBP-1 or -2 since they increased in
83 response to fasting, stress and cortisol injection (Siharath et al., 1996; Park et al., 2000; Kajimura
84 et al., 2003; Kelley et al., 2006; Kajimura and Duan, 2007). In salmon plasma/serum, three
85 IGFBPs are detected at 41, 28 and 22 kDa, respectively (Shimizu et al., 2000). We demonstrated,
86 through protein purification and cDNA cloning, that the 28- and 22-kDa IGFBPs were
87 co-orthologs of mammalian IGFBP-1 and named them IGFBP-1a and -1b, respectively (Shimizu
88 et al., 2005; 2011a). However, based on nomenclature proposed by Macqueen et al. (2013),
89 circulating salmon 28- and 22-kDa IGFBPs correspond to IGFBP-1a1 and -1b1, respectively.
90 Given their similar molecular weights and physiological regulation, the two circulating
91 low-molecular-weight IGFBPs in other fishes are likely also IGFBP-1 subtypes.

92 The presence of two subtypes of fish IGFBP-1 and their function were first shown in
93 zebrafish (*Danio rerio*; Kamei et al., 2008). Zebrafish IGFBP-1a and -1b are capable of inhibiting
94 proliferation of embryonic cells, demonstrating their inhibitory actions, consistent with
95 mammalian IGFBP-1. Kamei et al. (2008) proposed that although their IGF-inhibitory action
96 overlapped, they underwent subfunctional partitioning in terms of IGF-binding affinity, temporal
97 expression, and physiological response. We showed that salmon *igfbp-1* subtypes were
98 differentially expressed: *igfbp-1a* was widely distributed in many tissues while *igfbp-1b* was
99 almost exclusively expressed in the liver, suggesting spatially partitioned functions (Shimizu et
100 al., 2011a). Together this suggests that IGFBP-1 subtypes play pivotal roles in inhibiting
101 circulating IGF-I actions in fish.

102 Functional studies on fish IGFBP-1 have been done in zebrafish and carp (*Cyprinus*
103 *carpio*) using a morpholino knockdown approach (Kajimura et al., 2005; Kamei et al., 2008; Sun
104 et al., 2011). However, such analysis is restricted to developing fish embryos, and no studies have
105 examined roles of IGFBPs in postnatal growth in fish. This is mainly due to the lack of enough
106 purified IGFBPs. Purification of IGFBP-1 from serum is not practical since its circulating levels
107 are not high (Shimizu et al., 2005; 2011a). The aims of the present study were to produce

108 recombinant salmon IGFBP-1 subtypes using a bacterial expression system and test their effects
109 on IGF-regulated GH secretion from salmon pituitary cells in vitro.

110

111 **2. Materials and Methods**

112 *2.1. Cloning of open reading frames (ORFs) of masu salmon igfbp-1a and -1b*

113 Liver was collected from yearling masu salmon (*Oncorhynchus masou*) that had been fasted for 1
114 month at Nanae Freshwater Experimental Station, Field Science Center for Northern Biosphere,
115 Hokkaido University, Japan (Kameda-gun, Hokkaido, Japan). Total RNA was extracted from the
116 liver using Isogen (Nippon gene; Tokyo, Japan) and single-strand cDNA was reverse-transcribed
117 using SuperScript III (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions.

118 A primer set flanking the ORF of *igfbp-1a* (Table 1) was designed based on the cDNA
119 sequences of Chinook salmon (*O. tshawytscha*) and rainbow trout (*O. mykiss*) as reported in
120 Shimizu et al. (2011a). Reverse transcriptase (RT)-PCR was performed using AmpliTaq Gold®
121 360 Master Mix (Applied Biosystems, Foster City, CA) and a Veriti Thermal Cycler (Applied
122 Biosystems). PCRs consisted of 1 cycle of 95°C for 10 min; 35 cycles of 95°C for 30 sec, 60°C
123 for 30 sec, 72°C for 1 min; 1 cycle of 72°C for 7 min. PCR products were cloned into the pGEM-T
124 Easy Vector Systems (Promega, Madison, WI) and positive clones were sequenced as described
125 in Shimizu et al. (2011b).

126 Part of the ORF of *igfbp-1b* was amplified using a primer set designed similarly as
127 described above (Table 1). The complete ORF sequence of *igfbp-1b* was obtained by 5'- and
128 3'-RACE (rapid amplification of cDNA ends) using SMARTer™ RACE cDNA Amplification Kit
129 (Clontech, Palo Alto, CA) with gene-specific primers (Table 1).

130

131 *2.2. Subcloning into the expression vector*

132 Subcloning of cDNAs encoding the ORFs of *igfbp-1a* and *-1b* into the pET-32a(+) expression
133 vector (Novagen, Madison, WI) was performed by use of In-Fusion™ Advantage PCR Cloning
134 Kit (Clontech) according to the manufacturer's instructions and the methods described in Mizuta
135 et al. (2013). Briefly, the PCR reaction was performed with primers that contained adaptor
136 sequences corresponding to the terminal 15 bp sequences of the expression vector (Table 1).
137 PCRs consisted of 1 cycle of 98°C for 10 sec; 35 cycles of 98°C for 10 sec, 55°C for 5 sec, 72°C
138 for 2 min. The pET-32a(+) vector (1 ng) was linearized by an inverse PCR using PrimeSTAR®
139 Max Premix (Takara Bio, Shiga, Japan) and a primer set (10 μM each) complementary to the
140 vector (Table 1) in a 50-μl reaction. PCRs consisted of 1 cycle of 95°C for 10 sec; 35 cycles of

141 98°C for 10 sec, 50°C for 5 sec, 72°C for 15 sec. The PCR products were separated by 1.5%
142 agarose gel, purified and cloned into the pET32a(+) expression vector which provides
143 thioredoxin (Trx) and a His-tag (6 x His) at the N-terminal region of the resulting recombinant
144 protein as fusion partners. This construct was confirmed by sequencing.

145

146 *2.3. Expression of recombinant proteins*

147 The constructed plasmids (pET-Trx.His.rsIGFBP-1a and -1b) were transformed into the
148 *Escherichia coli* strain Rosetta-gamiTM B(DE3)pLysS (Novagen). Transformants were cultured
149 overnight in 3 ml Luria-Bertani (LB) medium containing ampicillin (50 µg/ml), tetracycline (12.5
150 µg/ml), kanamycin (15 µg/ml) and chloramphenicol (34 µg/ml) at 37°C and expression of
151 Trx.His.rsIGFBP-1a and -1b was induced by the addition of isopropyl-β-D-thiogalactoside
152 (IPTG) to a final concentration of 1 mM. The cell pellet collected after centrifugation was
153 resuspended with 5 ml of Bugbuster Protein Extraction Reagent (Novagen) containing 0.2%
154 lysonase and 1% cocktail protease inhibitor (Novagen) per 1 g of bacterial pellet.
155 Trx.His.rsIGFBP-1a and -1b appeared to be dominant in the insoluble fraction and were purified
156 by His-Bind Resin (Novagen) chromatography. After binding of the recombinant protein to
157 Ni-charged His-Bind Resin, the column was washed with a buffer containing 60 mM imidazole,
158 and the samples were subsequently eluted with a buffer containing 1 M imidazole. The elute was
159 dialyzed with ~1 L of 20 mM Tris-HCl (pH 8.0) containing 2% NaCl and 6 M urea and refolded
160 by gradually decreasing the urea concentration during dialysis.

161

162 *2.4. Enzymatic digestion and purification of recombinant proteins*

163 Refolded Trx.His.rsIGFBP-1a and -1b were digested by an enterokinase using Tag.offTM High
164 Activity rEK Kit (Novagen). Twenty micrograms of protein were mixed with 1 µl (1 unit)
165 enterokinase and incubated at room temperature for 20 h. Digested rsIGFBP-1a or -1b were
166 applied to reversed-phase high pressure liquid chromatography (HPLC) using a Vydac C-4
167 column (0.46 x 5 cm; Separation Group, Hesperia, CA). rsIGFBPs were separated from
168 undigested Trx.His.rsIGFBPs and Trx.His by a linear gradient of 0-80% acetonitrile in 0.1%
169 trifluoroacetic acid (TFA). Fractions were collected into low-adsorption 0.5 ml tubes (PGC
170 Scientifics, Frederick, MD). Acetonitrile and TFA were removed by N₂ evaporation from the
171 fractions and purified proteins were concentrated by semi-lyophilization. Protein concentrations
172 of purified rsIGFBP-1s were determined using the BCA Protein Assay Kit (Pierce Chemical,
173 Rockford, IL).

174

175 *2.5. Electrophoresis and ligand blotting*

176 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 3% stacking gel
177 and 12.5% separating gel was carried out. Samples were treated with an equal volume of the
178 sample buffer containing 2% SDS and 10% glycerol with or without 5% 2-mercaptoethanol at
179 85°C for 5 min. Gels were run in a solution of 50 mM Tris, 400 mM glycine and 0.1% SDS at 50
180 V in the stacking gel and at 100 V in the separating gel until the bromophenol blue dye front
181 reached the bottom of the gel. Gels were stained with 0.1% Coomassie Brilliant Blue (CBB) R250
182 (Bio-Rad, Hercules, CA). Molecular mass was estimated with Precision Marker (Bio-Rad).

183 Western ligand blotting with digoxigenin-labeled human IGF-I (DIG-hIGF-I) was
184 carried out as described in Shimizu et al. (2000). After electroblotting, the nitrocellulose
185 membrane was incubated with 10-50 ng/ml DIG-hIGF-I for 2 h at room temperature and then
186 incubated with an antibody against DIG, conjugated with horseradish peroxidase (Roche,
187 Indianapolis, IN) at a dilution of 1:1,500-2,500 for 1 h at room temperature. IGFBP was
188 visualized by use of enhanced Chemiluminescence (ECL) Prime Western blotting reagents
189 (Amersham Life Science, Arlington Heights, IL).

190

191 *2.6. Primary pituitary cell culture*

192 Primary pituitary cell culture was conducted according to methods of Ando et al. (2004) with
193 some modifications. Pituitaries were collected from 150 one-year-old masu salmon of both sexes
194 (fork length 21.0 ± 0.3 cm, body weight 88.8 ± 3.9 g; expressed as mean \pm SE of 10 fish) at Toya
195 Lake Station, Field Science Center for Northern Biosphere, Hokkaido University (Abuta-gun,
196 Hokkaido, Japan) in late November 2016. The experiment was carried out in accordance with
197 guidelines of the Hokkaido University Animal Care and Use Committee. The fish were
198 anesthetized with 3.3% 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan), decapitated, and
199 pituitaries were collected. The pituitaries were immersed in ice-cold RPMI containing 20 mM
200 HEPES (Gibco Invitrogen, Carlsbad, CA), 9 mM sodium bicarbonate, penicillin-streptomycin
201 (100U/ml) (Gibco Invitrogen) and fungizone (0.25 mg/ml) (Gibco Invitrogen), and brought back
202 to the laboratory. They were minced with a surgical blade and transferred to the above medium
203 supplemented with 0.1% collagenase (Gibco Invitrogen) and 3% bovine serum albumin (BSA,
204 Gibco Invitrogen). After incubation at 12°C for 20 h, cells were mechanically dissociated by
205 aspiration several times in a 10-ml pipette. They were washed twice with the RPMI medium
206 supplemented with Serum Replacement 2 (Sigma-Aldrich, Tokyo, Japan), which does not contain

207 steroid hormones, glucocorticoids or growth factors (Sigma-Aldrich). The dispersed cells were
208 immediately plated on 12-well culture plates (BD Biosciences, Franklin Lakes, NJ), pre-coated
209 with 50 µg/ml poly-D-lysine hydrobromide (Sigma, St. Louis, MO), at a density of 2.3×10^5
210 cells/1.3 ml medium/well ($n = 3$) and allowed to attach to the wells at 12°C for 48 h. After the
211 wells were washed with the medium, human IGF-I (GroPep Bioreagents, Thebarton, Australia)
212 was added to the medium and cells were further incubated for 24 h. After incubation, media were
213 collected and frozen at -80°C until assayed for GH. The cells were harvested by scraping them
214 twice in 0.7 ml of medium, collected by centrifugation at 5,000 rpm for 10 min, and frozen at
215 -80°C until use. Our preliminary experiments on the effect of incubation time (6, 12, 24, 48 and
216 72 h) after adding human IGF-I (hIGF-I; GroPep, Adelaide, Australia) suggested that incubation
217 for 24 or 48 h was optimal to capture the release of GH in response to hIGF-I treatment (data not
218 shown).

219

220 2.7. Immunoblotting

221 GH levels in the culture media were semi-quantified by immunoblotting using an antiserum
222 against GH. Purified chum salmon GH and antiserum against this GH were gifts from Dr.
223 Shunsuke Moriyama, Kitasato University, and Dr. Akihiko Hara, Hokkaido University,
224 respectively. The culture media (20 µl each) were separated by SDS-PAGE and electroblotted
225 onto a nitrocellulose membrane as described in Shimizu et al. (2000). The membrane was
226 incubated with anti-GH serum at a dilution of 1:50,000 for 2 h at room temperature and then with
227 goat anti-rabbit IgG-HRP conjugate (Bio-Rad) at a dilution of 1:20,000 for 1 h at room
228 temperature. Immunoreactive bands were visualized using ECL Prime Western blotting reagent.
229 Intensities of the GH bands were quantified using ImageJ program and normalized between blots
230 using purified GH as a reference.

231

232 2.8. Real-time quantitative PCR (qPCR)

233 Primers for qPCR of *gh* (Forward: 5' AATGGTCAGAAACGCCAACCAG 3'; Reverse: 5'
234 CAGCTGCTGAGAGTCATTGTCATC 3') were designed from a masu salmon *gh* cDNA
235 sequence (GenBank Accession No. X59762). Primer sequences for *ef-1a* (Forward: 5'
236 GAATCGGCCATGCCCGGTGAC 3'; Reverse: 5' GGATGATGACCTGAGCGGTG 3') were
237 from Shimomura et al. (2012). Both primer sets were designed to span an exon/exon boundary to
238 avoid amplification of genomic DNA. Reverse transcribed-PCR using these primer sets was
239 performed to prepare assay standards. PCR products run on 1.5% agarose gels were excised and

240 purified using QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA). Copy numbers of the
241 purified amplicons were calculated from the molecular weight of the amplicon and concentration.
242 The standard cDNAs were serially diluted from 1×10^7 to 3×10^2 copies. qPCR assays were
243 prepared using Power SYBR Green PCR Master Mix (Applied Biosystems) in a reaction volume
244 of 20 μ l with primer concentration of 100 nM and run on a 7300 Sequence Detector (Applied
245 Biosystems) using the manufacturer's recommended cycling conditions: 50°C for 2 min, 95°C for
246 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Measured values for *gh* were
247 normalized to those of *ef-1a*. Specificity of each primer pair was confirmed by the appearance of
248 a single peak in the dissociation curve and the lack of amplification for no template controls (data
249 not shown). The performance of qPCR was evaluated by calculating the amplification efficiencies
250 of the standard curves, which were within the range of 97-100%. The coefficients of
251 determination for the standard curves were between 0.99-1.00.

252

253 2.9. Statistical analysis

254 Results of the cell culture experiments were analyzed first by two-way ANOVA (IGF x IGFBP)
255 using the JMP program (SAS Institute Inc., Cary, NC). When significant effects were found,
256 differences were further identified by one-way ANOVA followed by Fisher's protected least
257 significant difference (PLSD) test. Differences among groups were considered significant at $P <$
258 0.05.

259

260 3. Results

261 3.1. cDNA cloning of masu salmon *igfbp-1a* and *-1b* ORFs

262 cDNAs for masu salmon *igfbp-1a* and *-1b* ORFs were cloned and their sequences deposited in
263 GenBank (Submission ID# 1983152). Masu salmon IGFBP-1a and -1b consist of 268 and 247
264 amino acid residues with estimated molecular weights of 25,988 and 24,127 Da, respectively.

265

266 3.2. Expression and purification of rsIGFBP-1a and -1b

267 Trx.His.rsIGFBP-1a and -1b were induced by adding IPTG to the culture medium and appeared
268 as 49- and 39-kDa bands, respectively, on SDS-PAGE under reducing conditions (Fig. 1). Both
269 Trx.His.rsIGFBP-1a and -1b were mainly found in the insoluble fraction, and were extracted
270 using 0.2% lysonate and solubilized in a buffer containing 6 M urea. They were separated from
271 bacterial proteins by Ni-affinity chromatography (data not shown) and refolded by gradually
272 reducing the urea concentration by dialysis. The refolded Trx.His.rsIGFBP-1a showed the ability

273 to bind DIG-IGF-I on ligand blotting (Fig. 2). The fusion partners (i.e. Trx and His-tag) were
274 cleaved from rsIGFBP-1a by enzymatic digestion using enterokinase (Fig. 2). rsIGFBP-1a was
275 further purified by reversed-phase HPLC using a C4 column (Fig. 3). Similarly,
276 Trs.His.rsIGFBP-1b was enzymatically cleaved (Fig. 4) and subjected to HPLC (Fig. 5). Purified
277 rsIGFBP-1a was detected as a primary band at 27 kDa and minor band at 29 kDa, while
278 rsIGFBP-1b appeared as broad bands around 22 kDa on SDS-PAGE under non-reducing
279 conditions (Fig. 6). By ligand blot, rsIGFBP-1a and -1b appeared at 28 and 22 kDa, respectively,
280 which corresponded to respective molecular weights of the intact proteins in the serum (Fig. 6).

281

282 3.3. Effects of rsIGFBP-1a and -1b on GH release from pituitary cells

283 Effects of hIGF-I and rsIGFBP-1a and -1b on GH release from cultured, adult masu salmon
284 pituitary cells were examined (Fig. 7a). hIGF-I alone increased GH release by 24 h. The addition
285 of rsIGFBP-1a diminished the IGF-induced GH release, whereas the addition of rsIGFBP-1b had
286 no significant effect. Neither rsIGFBP-1a nor -1b affected the GH release. Pituitary *gh* mRNA
287 levels were not affected by hIGF-I or rsIGFBP-1a (Fig. 7b). On the other hand, a significant
288 overall effect of IGFBP-1b on *gh* mRNA ($P = 0.011$) was apparent by two-way ANOVA (Fig. 7b).

289

290 4. Discussion

291 Production of recombinant protein is useful for functional analysis. Expression of recombinant
292 IGFBPs has been primarily conducted with zebrafish (Duan et al., 1999; Kamei et al., 2008; Zhou
293 et al., 2008; Dai et al., 2010). For example, Kamei et al. (2008) expressed and purified
294 recombinant zebrafish IGFBP-1a and -1b with fusion partners Myc and His-tag and showed that
295 both IGFBPs inhibited the IGF-I-induced cell proliferation of cultured zebrafish embryonic cells.
296 Also, recombinant zebrafish IGFBP-2 and -5 were shown to inhibit IGF actions (Duan et al.,
297 1999; Zhou et al., 2008; Dai et al., 2010). However, there are only a few studies in other fish
298 species that have characterized IGFBPs (Bauchat et al., 2001; Shimizu et al., 2003; Zhou et al.,
299 2013). This is mainly due to the lack of purified/recombinant protein. The present study produced
300 recombinant salmon (rs) IGFBP-1 subtypes using a bacterial expression system and examined
301 their effects on GH release from salmon pituitary cells.

302 We expressed rsIGFBP-1 with fusion partners, thioredoxin (Trx) and His-tag, using
303 the pET-32a(+) expression vector. Trx is often used as a fusion partner of recombinant protein
304 since it increases translation efficiency and solubility of recombinant protein (LaVallie et al.,
305 1993). In addition, it acts as a molecular chaperon to help the protein fold correctly and form

306 disulfide bonds (Kern et al., 2003). Expressing Trx as a fusion partner was effective to produce a
307 soluble, correctly folded rat IGFBP-4 (Chelius et al. 2001). In the present study, however, Trx was
308 not effective to retain the recombinant protein in the soluble fraction. On the other hand, the
309 Trx.His.IGFBP-1s showed an ability to bind the labeled IGF-I, as confirmed by ligand blotting,
310 indicating that correct protein folding was achieved with the presence of Trx. Protein folding is
311 one of the critical steps for producing functional recombinant protein, especially for proteins with
312 disulfide bonds. Since Trx.His.IGFBP-1s were found in the insoluble fraction, addition of 6 M
313 urea was necessary to solubilize them. With urea present, the disulfide bonds of the IGFBP-1s
314 were dissociated. IGFBP-1 has 12 and 6 cysteine residues in the N- and C-terminal domains,
315 respectively, and forms intra-domain disulfide bonds (Neumann and Bach, 1999). Our attempts to
316 produce rsIGFBP-1s with only His-tags using the pET-16b vector were unsuccessful due to the
317 formation of a precipitate during dialysis, which was probably due to incorrect refolding without
318 Trx (data not shown). Thus, addition of Trx was indeed necessary to achieve correct protein
319 refolding. Indeed, Wilkinson et al. (2004) reported that the strategy to express rsIGF-II using the
320 pET32a first in the insoluble fraction and then refold it with the aid of Trx was effective to obtain
321 a high yield of functional protein. Thus, the procedure established in this study is one good way to
322 obtain these functional recombinant proteins.

323 We prepared recombinant fish IGFBP for the first time without a fusion partner. The
324 fusion partners Trx and His-tag were enzymatically cleaved from rsIGFBP-1 using an
325 enterokinase and rsIGFBP-1 was purified by reversed-phase HPLC. Although Trx.His.IGFBP-1s
326 had the ability to bind IGF-I, it is preferable not to have fusion partners for functional analyses.
327 An enterokinase cleavage site in the vector was used to separate rsIGFBP-1s from the fusion
328 partners. However, an apparent non-specific digestion of rsIGFBP-1b occurred when the
329 recommended amount of enterokinase was used (data not shown). Applying half the amount of
330 enterokinase protected rsIGFBP-1s but resulted in lower cleavage efficiencies. The recovery rate
331 of rsIGFBP-1b from Trx.His.IGFBP-1b after HPLC was 10% while it was 22% for rsIGFBP-1a.
332 Further optimization of the production of rsIGFBP-1s, such as using another enzyme recognition
333 site, would increase the yield.

334 Inhibition of pituitary GH production and secretion by circulating IGF-I is an
335 important negative feedback loop of the somatotrophic system (Ohlsson et al., 2009). In vitro
336 experiments in mammals and fishes have demonstrated that IGF-I directly acts on the pituitary
337 cells (Pérez-Sánchez et al., 1992; Fruchtman et al., 2000, 2002; Luckenbach et al., 2010). In the
338 present study, IGF-I alone unexpectedly increased GH release from the pituitary cells in the

339 present study. This may be due to the season and/or stage of the masu salmon used in the
340 experiment. Pituitaries were collected from one-year-old male and female masu salmon in late
341 November. These fish were likely under a transition from immature to early maturing stage
342 (Furukuma et al., 2008). Previous studies showing the inhibitory effects of IGF on GH release
343 used pituitary cells from immature fish (Pérez-Sánchez et al., 1992; Fruchtman et al., 2000, 2002;
344 Luckenbach et al., 2010). In addition, fish were sampled in early winter when growth was reduced.
345 Thus, during the early reproductive stage and/or non-growing season, the negative feedback loop
346 between GH and IGF-I could be diminished. This of course is speculative and future studies are
347 necessary to examine the stage/season dependent IGF-I action on GH release.

348 The present study examined for the first time, combined effects of IGF-I and
349 rsIGFBP-1a or -1b on GH release in vitro in fish. Whether or not IGF-I can modulate GH
350 production and secretion in vivo may depend on the type of IGFBP carrying the IGF-I.
351 Circulating IGFBP-1 could inhibit the interaction of IGF-I with IGF-receptors on the pituitary
352 cells. Results of the present study suggest that rsIGFBP-1a is capable of modulating IGF-I action
353 at the pituitary level. When rsIGFBP-1a was added in combination with IGF-I to the culture
354 medium, increased GH release was restored to the basal level, suggesting that effects of IGF-I
355 were inhibited by IGFBP-1a. Our finding is in accord with the suggestion by Fruchtman et al.
356 (2002) that IGFBPs play a significant role in modulating the action of IGF-I on GH release. On
357 the other hand, IGFBP-1b had no significant inhibitory effect on IGF-I action. These disparate
358 effects may be due to differences in IGF-binding affinity between rsIGFBP-1a and -1b. In
359 zebrafish, IGFBP-1a had a higher affinity for IGFs than did IGFBP-1b (Kamei et al., 2008). We
360 did not specifically measure the affinity of rsIGFBP-1a and -1b for IGFs in the present study.
361 However, when the same amount of rsIGFBP-1a and -1b was loaded on the gel and analyzed by
362 ligand blotting, the band intensity of IGFBP-1a was stronger than that of IGFBP-1b, suggesting
363 IGFBP-1a has a higher affinity for IGF-I. Kamei et al. (2008) proposed that zebrafish IGFBP-1a
364 and -1b partitioned IGF-inhibitory actions temporally and physiologically. In agreement with this,
365 our results support subfunctionalization of the two salmon IGFBP-1 subtypes, in terms of their
366 degree of IGF-inhibitory action.

367 Post-translational modification of proteins, such as phosphorylation and glycosylation,
368 occur in IGFBPs and may modulate their actions (Firth and Baxter, 2002; Forbes et al., 2012). It is
369 of note that rsIGFBP-1a and -1b produced in the bacterial system are unlikely to be
370 phosphorylated. Mammalian IGFBP-1 is usually highly phosphorylated and the degree of
371 phosphorylation affects its affinity for IGFs, being higher for the phosphorylated form in humans

372 (Jones et al., 1991). Conversely, dephosphorylation of rat IGFBP-1 did not reduce its IGF-binding
373 affinity (Peterkofsky et al., 1998). Since the deduced amino acid sequences of salmon IGFBP-1a
374 and -1b possess potential phosphorylation sites in the L-domain (Shimizu et al., 2005, 2011a),
375 results of the present study using non-phosphorylated rsIGFBP-1s need to be interpreted with
376 caution.

377 IGFbps can exhibit IGF-independent actions on cell growth through interacting with
378 the cell surface and/or translocating into the nucleus (Firth and Baxter, 2002; Forbes et al., 2012).
379 Mammalian IGFBP-1 has an Arg-Gly-Asp (RGD) motif that is a cell surface $\alpha 5\beta 1$ integrin
380 binding site. In contrast, there is no RGD motif in salmon or other fish IGFBP-1s (Kamei et al.,
381 2008; Shimizu et al., 2011a). In addition, neither mammalian nor fish IGFBP-1s have a nuclear
382 localization sequence. These structural features suggest that fish IGFBP-1s function through
383 modulation of IGF-I actions. Supporting this, when rsIGFBP-1a or -1b was added to the culture
384 medium in the absence of IGF-I, they had no effect on GH release. However, there was an overall
385 effect of rsIGFBP-1b on *gh* mRNA, which may suggest its direct action. More experiments are
386 needed to address whether or not salmon IGFBP-1s have IGF-independent actions.

387 In summary, this study is the first to produce and purify rsIGFBP-1a and -1b without
388 fusion partners in a bacterial expression system. The rsIGFBP-1s demonstrated an ability to bind
389 IGF-I and rsIGFBP-1a specifically exhibited IGF-inhibitory action in our salmon pituitary cell
390 culture system. The availability of rsIGFBP-1a and -1b enable us to further analyze their
391 physiological functions. In addition, these recombinant proteins should be useful as antigens for
392 raising specific antisera and as standards for immunoassays.

393

394 **Acknowledgement**

395 We thank Akihiko Hara, Faculty of Fisheries Sciences, Hokkaido University, and Shunsuke
396 Moriyama, Kitasato University School of Marine Biosciences, for providing antiserum against
397 chum salmon GH and purified chum salmon GH, respectively. We also thank Nanae Freshwater
398 Experimental Station and Toya Lake Station, Field Science Center for Northern Biosphere,
399 Hokkaido University, Japan, for providing experimental fish. We extend our thanks to J. Adam
400 Luckenbach, Northwest Fisheries Science Center, NOAA Fisheries, for correcting the English in
401 the manuscript. This work was supported by JSPS KAKENIH Grant Numbers JP24580256 and
402 JP16H04966.

403

404 **References**

405 Ando, H., Swanson, P., Kitani, T., Koide, N., Okada, H., Ueda, H., Urano, A., 2004. Synergistic
406 effects of salmon gonadotropin-releasing hormone and estradiol-17 beta on gonadotropin
407 subunit gene expression and release in masu salmon pituitary cells in vitro. *Gen. Comp.*
408 *Endocrinol.* 137, 109-121.

409 Bauchat, J.R., Busby, W.H., Jr., Garmong, A., Swanson, P., Moore, J., Lin, M., Duan, C., 2001.
410 Biochemical and functional analysis of a conserved IGF-binding protein isolated from
411 rainbow trout (*Oncorhynchus mykiss*) hepatoma cells. *J. Endocrinol.* 170, 619-628.

412 Chelius, D., Baldwin, M.A., Lu, X., Spencer, E.M., 2001. Expression, purification and
413 characterization of the structure and disulfide linkages of insulin-like growth factor
414 binding protein-4. *J. Endocrinol.* 168, 283-296.

415 Dai, W., Kamei, H., Zhao, Y., Ding, J., Du, Z., Duan, C., 2010. Duplicated zebrafish insulin-like
416 growth factor binding protein-5 genes with split functional domains: evidence for
417 evolutionarily conserved IGF binding, nuclear localization, and transactivation activity.
418 *FASEB J.* 24, 2020-2029.

419 Daughaday, W.H., Rotwein, P., 1989. Insulin-like growth factors I and II. Peptide, messenger
420 ribonucleic acid and gene structures, serum, and tissue concentrations. *Endocr. Rev.* 10,
421 68-91.

422 Daza, D.O., Sundstrom, G., Bergqvist, C.A., Duan, C.M., Larhammar, D., 2011. Evolution of the
423 insulin-like growth factor binding protein (IGFBP) family. *Endocrinology* 152,
424 2278-2289.

425 Duan, C., Ding, J., Li, Q., Tsai, W., Pozios, K., 1999. Insulin-like growth factor binding protein 2
426 is a growth inhibitory protein conserved in zebrafish. *Proc. Natl. Acad. Sci. USA* 96,
427 15274-15279.

428 Firth, S.M., Baxter, R.C., 2002. Cellular actions of the insulin-like growth factor binding proteins.
429 *Endocr. Rev.* 23, 824-854.

430 Forbes, B.E., McCarthy, P., Norton, R.S., 2012. Insulin-like growth factor binding proteins: a
431 structural perspective. *Front. Endocrinol.* 3, 38.

432 Fruchtmann, S., Jackson, L., Borski, R., 2000. Insulin-like growth factor I disparately regulates
433 prolactin and growth hormone synthesis and secretion: studies using the teleost pituitary
434 model. *Endocrinology* 141, 2886-2894.

435 Fruchtmann, S., McVey, D.C., Borski, R.J., 2002. Characterization of pituitary IGF-I receptors:
436 modulation of prolactin and growth hormone. *Am. J. Physiol. Regul. Integ. Comp.*
437 *Physiol.* 283, R468-R476.

438 Furukuma, S., Onuma, T., Swanson, P., Luo, Q., Koide, N., Okada, H., Urano, A., Ando, H., 2008.
439 Stimulatory effects of insulin-like growth factor 1 on expression of gonadotropin subunit
440 genes and release of follicle-stimulating hormone and luteinizing hormone in masu
441 salmon pituitary ceft early in gametogenesis. *Zool. Sci.* 25, 88-98.

442 Jones, J.I., D'Ercole, A.J., Camacho-Hubner, C., Clemmons, D.R., 1991. Phosphorylation of
443 insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: effects on
444 affinity for IGF-I. *Proc. Natl. Acad. Sci. USA* 88, 7481-7485.

445 Kajimura, S., Aida, K., Duan, C., 2005. Insulin-like growth factor-binding protein-1 (IGFBP-1)
446 mediates hypoxia-induced embryonic growth and developmental retardation. *Proc. Natl.*
447 *Acad. Sci. USA* 102, 1240-1245.

448 Kajimura, S., Duan, C., 2007. Insulin-like growth factor-binding protein-1: an evolutionarily
449 conserved fine tuner of insulin-like growth factor action under catabolic and stressful
450 conditions. *J. Fish Biol.* 71, 309-325.

451 Kajimura, S., Hirano, T., Visitacion, N., Moriyama, S., Aida, K., Grau, E.G., 2003. Dual mode of
452 cortisol action on GH/IGF-I/IGF binding proteins in the tilapia, *Oreochromis*
453 *mossambicus*. *J. Endocrinol.* 178, 91-99.

454 Kamei, H., Lu, L., Jiao, S., Li, Y., Gyrupe, C., Laursen, L.S., Oxvig, C., Zhou, J., Duan, C., 2008.
455 Duplication and diversification of the hypoxia-inducible IGFBP-1 gene in zebrafish.
456 *PLoS One* 3, e3091.

457 Kelley, K.M., Haigwood, J.T., Perez, M., Galima, M.M., 2001. Serum insulin-like growth factor
458 binding proteins (IGFBPs) as markers for anabolic/catabolic condition in fishes. *Comp.*
459 *Biochem. Physiol. B* 129, 229-236.

460 Kelley, K.M., Price, T.D., Galima, M.M., Sak, K., Reyes, J.A., Zepeda, O., Hagstrom, R., Truong,
461 T.A., Lowe, C.G., 2006. Insulin-like growth factor-binding proteins (IGFBPs) in fish:
462 beacons for (disrupted) growth endocrine physiology, in: Reinecke, M., Zacccone, G.,
463 Kapoor, B.G. (Eds.), *Fish Endocrinology*. Science Publishers, Enfield, New Hampshire,
464 pp. 167-195.

465 Kern, R., Malki, A., Holmgren, A., Richarme, G., 2003. Chaperone properties of *Escherichia coli*
466 thioredoxin and thioredoxin reductase. *Biochem. J.* 371, 965-972.

467 LaVallie, E.R., Diblasio, E.A., Kovacic, S., Grant, K.L., Schendel, P.F., McCoy, J.M., 1993. A
468 Thioredoxin Gene Fusion Expression System That circumvents inclusion body formation
469 in the *E. coli* cytoplasm. *Biotechnology* 11, 187-193.

470 Le Roith, D., Bondy, C., Yakar, S., Liu, J.L., Butler, A., 2001. The somatomedin hypothesis: 2001.

471 Endocr. Rev. 22, 53-74.

472 Lee, P.D., Conover, C.A., Powell, D.R., 1993. Regulation and function of insulin-like growth
473 factor-binding protein-1. Proc. Soc. Exp. Biol. Med. 204, 4-29.

474 Lee, P.D., Giudice, L.C., Conover, C.A., Powell, D.R., 1997. Insulin-like growth factor binding
475 protein-1: recent findings and new directions. Proc. Soc. Exp. Biol. Med. 216, 319-357.

476 Luckenbach, J.A., Dickey, J.T., Swanson, P., 2010. Regulation of pituitary GnRH receptor and
477 gonadotropin subunits by IGF1 and GnRH in prepubertal male coho salmon. Gen. Comp.
478 Endocrinol. 167, 387-396.

479 Macqueen, D.J., de la Serrana, D.G., Johnston, I.A., 2013. Evolution of ancient functions in the
480 vertebrate insulin-like growth factor system uncovered by study of duplicated salmonid
481 fish genomes. Mol. Biol. Evol. 30, 1060-1076.

482 Mizuta, H., Luo, W.S., Ito, Y., Mushirobira, Y., Todo, T., Hara, A., Reading, B.J., Sullivan, C.V.,
483 Hiramatsu, N., 2013. Ovarian expression and localization of a vitellogenin receptor with
484 eight ligand binding repeats in the cutthroat trout (*Oncorhynchus clarki*). Comp. Biochem.
485 Physiol. B 166, 81-90.

486 Neumann, G.M., Bach, L.A., 1999. The N-terminal disulfide linkages of human insulin-like
487 growth factor-binding protein-6 (hIGFBP-6) and hIGFBP-1 are different as determined
488 by mass spectrometry. J. Biol. Chem. 274, 14587-14594.

489 Ohlsson, C., Mohan, S., Sjogren, K., Tivesten, A., Isgaard, J., Isaksson, O., Jansson, J.O.,
490 Svensson, J., 2009. The role of liver-derived insulin-like growth factor-I. Endocr. Rev. 30,
491 494-535.

492 Park, R., Shepherd, B.S., Nishioka, R.S., Grau, E.G., Bern, H.A., 2000. Effects of homologous
493 pituitary hormone treatment on serum insulin-like growth-factor-binding proteins
494 (IGFBPs) in hypophysectomized tilapia, *Oreochromis mossambicus*, with special
495 reference to a novel 20-kDa IGFBP. Gen. Comp. Endocrinol. 117, 404-412.

496 Pérez-Sánchez, J., Weil, C., Le Bail, P.Y., 1992. Effects of human insulin-like growth factor-I on
497 release of growth hormone by rainbow trout (*Oncorhynchus mykiss*) pituitary cells. J.
498 Exp. Zool. 262, 287-290.

499 Peterkofsky, B., Gosiewska, A., Wilson, S., Kim, Y.R., 1998. Phosphorylation of rat insulin-like
500 growth factor binding protein-1 does not affect its biological properties. Arch. Biochem.
501 Biophys. 357, 101-110.

502 Rajaram, S., Baylink, D.J., Mohan, S., 1997. Insulin-like growth factor-binding proteins in serum
503 and other biological fluids: regulation and functions. Endocr. Rev. 18, 801-831.

504 Shimizu, M., Dickey, J.T., Fukada, H., Dickhoff, W.W., 2005. Salmon serum 22 kDa insulin-like
505 growth factor-binding protein (IGFBP) is IGFBP-1. *J. Endocrinol.* 184, 267-276.

506 Shimizu, M., Kishimoto, K., Yamaguchi, T., Nakano, Y., Hara, A., Dickhoff, W.W., 2011a.
507 Circulating salmon 28- and 22-kDa insulin-like growth factor binding proteins (IGFBPs)
508 are co-orthologs of IGFBP-1. *Gen. Comp. Endocrinol.* 174, 97-106.

509 Shimizu, M., Suzuki, S., Horikoshi, M., Hara, A., Dickhoff, W.W., 2011b. Circulating salmon
510 41-kDa insulin-like growth factor binding protein (IGFBP) is not IGFBP-3 but an
511 IGFBP-2 subtype. *Gen. Comp. Endocrinol.* 171, 326-331.

512 Shimizu, M., Swanson, P., Fukada, H., Hara, A., Dickhoff, W.W., 2000. Comparison of extraction
513 methods and assay validation for salmon insulin-like growth factor-I using commercially
514 available components. *Gen. Comp. Endocrinol.* 119, 26-36.

515 Shimizu, M., Swanson, P., Hara, A., Dickhoff, W.W., 2003. Purification of a 41-kDa insulin-like
516 growth factor binding protein from serum of chinook salmon, *Oncorhynchus tshawytscha*.
517 *Gen Comp Endocrinol* 132, 103-111.

518 Shimomura, T., Nakajima, T., Horikoshi, M., Iijima, A., Urabe, H., Mizuno, S., Hiramatsu, N.,
519 Hara, A., Shimizu, M., 2012. Relationships between gill Na^+,K^+ -ATPase activity and
520 endocrine and local insulin-like growth factor-I levels during smoltification of masu
521 salmon (*Oncorhynchus masou*). *Gen. Comp. Endocrinol.* 178, 427-435.

522 Siharath, K., Kelley, K.M., Bern, H.A., 1996. A low-molecular-weight (25-kDa) IGF-binding
523 protein is increased with growth inhibition in the fasting striped bass, *Morone saxatilis*.
524 *Gen. Comp. Endocrinol.* 102, 307-316.

525 Sun, C.F., Tao, Y., Jiang, X.Y., Zou, S.M., 2011. IGF binding protein 1 is correlated with
526 hypoxia-induced growth reduce and developmental defects in grass carp
527 (*Ctenopharyngodon idellus*) embryos. *Gen. Comp. Endocrinol.* 172, 409-415.

528 Wheatcroft, S.B., Kearney, M.T., 2009. IGF-dependent and IGF-independent actions of
529 IGF-binding protein-1 and -2: implications for metabolic homeostasis. *Trends Endocr.*
530 *Met.* 20, 153-162.

531 Wilkinson, R.J., Elliott, P., Carragher, J.F., Francis, G., 2004. Expression, purification, and in
532 vitro characterization of recombinant salmon insulin-like growth factor-II. *Protein Expres*
533 *Purif.* 35, 334-343.

534 Zhou, J., Li, W., Kamei, H., Duan, C., 2008. Duplication of the IGFBP-2 gene in teleost fish:
535 protein structure and functionality conservation and gene expression divergence. *PLoS*
536 *One* 3, e3926.

537 Zhou, J.F., Xiang, J.H., Zhang, S.C., Duan, C.M., 2013. Structural and functional analysis of the
538 amphioxus IGFBP gene uncovers ancient origin of IGF-independent functions.
539 Endocrinology 154, 3753-3763.
540

541 **Figure legends**

542 Fig. 1. SDS-PAGE of extracts of bacterial cells transformed with pET32a-Trx.His.IGFBP-1a (left
543 panel) and -1b (right panel) before (-) and after (+) addition of IPTG to the culture medium.
544 Bacterial proteins induced by IPTG were fractionated into soluble and insoluble fractions via the
545 Bugbuster Protein Extraction Kit. SDS-PAGE was run under reducing conditions. Migration
546 positions of the molecular weight marker are indicated by arrowheads on the left. Arrowheads on
547 the right indicate proteins induced by IPTG and their approximate molecular weights.

548

549 Fig. 2. SDS-PAGE and ligand blotting of recombinant IGFBP-1a before (-) and after (+) digestion
550 by enterokinase (EK). Twenty micrograms of Trx.His.rsIGFBP-1a was incubated with 1 unit of
551 EK at room temperature for 20 h. They were separated by 12.5% SDS-PAGE under non-reducing
552 conditions and stained with Coomassie Brilliant Blue (CBB; left panel). The same samples were
553 electroblotted onto a nitrocellulose membrane after SDS-PAGE and subjected to ligand blotting
554 (LB; right panel) using digoxigenin-labeled human IGF-I (50 ng) and antiserum against
555 digoxigenin (1:20,000). Arrowheads indicate migration positions and molecular weights of
556 undigested Trx.His.rsIGFBP-1a and digested rsIGFBP-1a, respectively.

557

558 Fig. 3. Elution profiles of rsIGFBP-1a on reversed-phase HPLC. The digested sample was applied
559 to a Vydac c-4 column and eluted by a linear gradient of 0-80% acetonitrile in 0.1% TFA
560 (indicated by the dashed line). Numbers indicate fractions collected for analysis by ligand blotting
561 (inset). Arrowheads on the ligand blotting indicate migration positions of undigested (42 kDa)
562 and digested (28 kDa) recombinant IGFBP-1a.

563

564 Fig. 4. SDS-PAGE and ligand blotting of recombinant IGFBP-1b before (-) and after (+) digestion
565 by enterokinase (EK). Twenty micrograms of Trx.His.rsIGFBP-1b was incubated with 1 unit of
566 EK at room temperature for 20 h. They were separated by 12.5% SDS-PAGE under non-reducing
567 conditions and stained with Coomassie Brilliant Blue (CBB; left panel). The same samples were
568 electroblotted onto a nitrocellulose membrane after SDS-PAGE and subjected to ligand blotting
569 (LB; right panel) using digoxigenin-labeled human IGF-I (50 ng) and antiserum against
570 digoxigenin (1:20,000). Arrowheads indicate migration positions and molecular weights of
571 undigested Trx.His.rsIGFBP-1b and digested rsIGFBP-1b, respectively.

572

573 Fig. 5. Elution profiles of rsIGFBP-1b on reversed-phase HPLC. The digested sample was

574 applied to a Vydac c-4 column and eluted by a linear gradient of 0-80% acetonitrile in 0.1% TFA
575 (indicated by the dashed line). Numbers indicate fractions collected for analysis by ligand blotting
576 (inset). Arrowheads on the ligand blotting indicate migration positions of undigested (35 kDa)
577 and digested (22 kDa) recombinant IGFBP-1b.

578

579 Fig. 6. SDS-PAGE and ligand blotting of purified rsIGFBP-1a and -b. Eight micrograms of
580 purified protein was loaded onto 12.5% gels and electrophoresed under non-reducing conditions.
581 One gel was stained with Coomassie Brilliant Blue (CBB; left panel) and the other was subjected
582 to ligand blotting (LB; right panel) using digoxigenin-labeled human IGF-I (50 ng) and antiserum
583 against digoxigenin (1:20,000). Arrowheads indicate migration positions and molecular weights
584 of purified proteins. Serum: masu salmon serum.

585

586 Fig. 7. Effects of rsIGFBP-1a and -1b on GH release by cultured masu salmon pituitary cells into
587 the media (a) and *gh* mRNA in the pituitary cells (b). Pituitary cells from one-year-old male and
588 female masu salmon were pre-cultured for 2 days and incubated for 24 h without (-) or with (+)
589 human IGF-I and rsIGFBP-1a or rsIGFBP-1b. Asterisks indicate overall effects. Symbols sharing
590 the same letters are not significantly different from each other.

Table 1

Primer sequences used for cDNA cloning and subcloning

Primer Name	Primer sequence (5'-3')	Direction	Use
IGFBP-1a ORF F	CTTCCTTATTCAGTCTCTCCTTAT	Foward	RT PCR
IGFBP-1a ORF R	TGCATGCGTGCGTGTCT	Reverse	RT PCR
IGFBP-1b ORF F	GAGATGCTTGGATTATATAAGAAGTTG	Forward	RT PCR
IGFBP-1b ORF R	TTAGAGCTCCAGCTGGCACT	Reverse	RT PCR
IGFBP-1b GSP F	GACCTCAGCTCCCTGCCCTACCTCCT	Forward	3'-RACE
IGFBP-1b GSP R	CAGCTCAATGTGGCAGGGCCCCTG	Reverse	5'-RACE
pET32a F	GCCATGGCTGATATCGGATC	Foward	Inverse PCR
pET32a F	CTTGTCGTCGTCGTCGGTAC	Reverse	Inverse PCR
pET32a-IGFBP-1a F	<u>GACGACGACGACAAGTCCCCGGTGTAGCGCAA</u>	Forward	InFusion PCR
pET32a-IGFBP-1a R	<u>GATATCAGCCATGGCTCAGTGGTTGATCTCATAAGCGCA</u>	Reverse	InFusion PCR
pET32a-IGFBP-1b F	<u>GACGACGACGACAAGTCCCCGTGGTAGGTCCA</u>	Forward	InFusion PCR
pET32a-IGFBP-1b R	<u>GATATCAGCCATGGCTTAGTGAGTGAGCTCCTGCTGACA</u>	Reverse	InFusion PCR

ORF: open reading frame; GSP: gene-specific primer. Underlines are sequences complement to pET-32a













