

No evidence for enhanced parvalbumin concentration in light muscle of transgenic coho salmon (*Oncorhynchus kisutch*)

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Abstract The expression of parvalbumin, a major allergenic protein of fish muscle, was determined in rapidly growing transgenic coho salmon (*Oncorhynchus mykiss*) containing the growth hormone (GH) gene construct OnMTGH1. Three different methods for parvalbumin analysis were used: (1) measurement of the mRNA concentration by real-time RT-PCR; (2) isoelectric focusing of sarcoplasmic proteins; (3) assessment of amount of heat-stable, Ca^{2+} -binding sarcoplasmic proteins by measuring absorbance spectra of “stains-all”-protein complexes. Compared to non-transgenic coho salmon, no indication for enhanced expression of parvalbumin in transgenic fish was found either at the mRNA or at the protein level.

Keywords Transgenic fish · Coho salmon · Parvalbumin

Introduction

Food safety of transgenic fish may depend on the gene product as such and/or potential pleiotropic effects. Pleiotropic effects are defined as indirect changes of the phenotype (metabolism, composition, appearance) in consequence of a genetic modification. Growth enhancement is in many cases the aim for genetically modifying fish-like

carp, salmon or tilapia fish growth hormone gene constructs [1]. Up to now, no evidence was found that enhanced amounts of fish growth hormone in the fillet may be a risk for consumers [2].

However, pleiotropic effects have been described for transgenic coho salmon containing the GH gene construct OnMTGH1 [3–5]. Enhanced concentration of parvalbumin mRNA has been detected by subtractive hybridisation [6]. As parvalbumin is considered to be a major human allergen in food fish, consumption of transgenic coho salmon could thus be associated with a potential risk for human health.

We have compared expression of parvalbumin in the fillet of transgenic and non-transgenic fish by three different techniques: (1) measurement of the mRNA concentration by real-time RT-PCR; (2) determination of the patterns obtained by isoelectric focusing of sarcoplasmic proteins; (3) assessment of amount of heat-stable, Ca^{2+} -binding proteins by measuring absorbance spectra of “stains-all”-protein complexes.

Materials and methods

Fish husbandry and samples

Portions of light muscle of 36 specimens of size-matched transgenic and non-transgenic coho salmon (*Oncorhynchus kisutch*) were sampled in September 2005 and shipped as frozen material by air-cargo from Vancouver to Hamburg for further analysis. Details of the sex, size and weight of the fish are given in Table 1. The transgenic fish are from strain M77 which contains the OnMTGH1 sockeye salmon GH1 gene construct [5, 7]. Transgenic and non-transgenic salmon were fed to satiation three times per day, with the former having appetite and daily growth rate elevated more

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Table 1 Biological data of transgenic and non-transgenic coho salmon

Fish #	Genotype/tank/sex	Weight (g)	Length (cm)
Transgenic			
1	2004 T/IV/M	68.4	16.9
2	2004 T/IV/M	70.9	16.9
3	2004 T/IV/F	56.6	15.9
4	2004 T/IV/F	92.7	18.8
5	2004 T/IV/M	85.9	18.1
6	2004 T/IV/M	53.9	15.8
7	2004 T/IV/F	85.5	17.9
8	2004 T/IV/M	69.5	16.8
9	2004 T/IV/F	93.7	18.8
10	2004 T/IV/M	60.2	16.3
11	2004 T/IV/F	70.1	16.9
12	2004 T/IV/F	78.0	17.8
Non-transgenic			
13	2003 NT/V/M	67.3	17.6
14	2003 NT/V/M	76.2	18.1
15	2003 NT/V/M	77.5	18.5
16	2003 NT/V/M	92.3	19.5
17	2003 NT/V/M	88.7	19.1
18	2003 NT/V/F	76.6	18.1
19	2003 NT/V/F	108.4	20.8
20	2003 NT/V/F	61.0	17.2
21	2003 NT/V/F	87.8	18.8
22	2003 NT/V/M	63.5	17.5
23	2003 NT/V/F	111.3	20.3
24	2003 NT/V/F	85.6	17.5
Transgenic, ration-fed			
25	2003 TR/VI/F	113.6	22.0
26	2003 TR/VI/F	79.2	19.8
27	2003 TR/VI/F	51.2	17.5
28	2003 TR/VI/F	63.1	18.1
29	2003 TR/VI/M	66.2	18.2
30	2003 TR/VI/M	75.8	18.8
31	2003 TR/VI/F	76.7	19.0
32	2003 TR/VI/F	68.2	18.2
33	2003 TR/VI/M	58.7	17.3
34	2003 TR/VI/M	58.3	18.4
35	2003 TR/VI/M	78.7	19.7
36	2003 TR/VI/M	62.9	18.4

than fivefold over controls [8]. Ration-fed transgenic salmon were fed a restricted feed intake equal to the amount of food consumed by non-transgenic controls, and thus grew at a control rate. Non-transgenic and ration-fed (growth-restricted) transgenic salmon were 20 months of age (post-fertilisation), whereas transgenic salmon were 8 months of age to allow size matching with the control groups. All fish were reared in fresh well water at 10 °C.

Analytical methods

DNA analysis

DNA was extracted from muscle tissue using the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim). Quantification of DNA and PCR-SSCP (single-strand conformation polymorphism analysis) were performed according to [9] by amplifying a growth hormone gene sequence of ~450 bp.

Messenger RNA analysis by quantitative RT-PCR

The RNeasy Mini Kit (QIAGEN, Hilden, Germany) was used for isolation of RNA following the protocol for isolation of total RNA from muscle tissue as described in the handbook delivered with the kit. The procedure included a proteinase K digestion step and on-column DNase treatment. Ten to 20 mg of minced muscle was used as the starting material. The amount of extracted RNA was determined by reading the OD at 260 nm and calculating the RNA concentration (ng/μl) by multiplying the OD with the factor of 40.

Real-time PCR with the Light Cycler (RT-LC)

The C_T -value (number of cycle when a preset threshold of fluorescence is crossed) for parvalbumin mRNA related to concentration of extracted total RNA was determined with a two-step RT-PCR. In the first step, mRNA was transcribed into cDNA using the Sensiscript® RT Kit (QIAGEN) with primers IFF 156/IFF 157 being specific for a parvalbumin gene [9]. Assay conditions: volume, 20 μl; primer concentration, 0.5 μM (IFF156/IFF 157); RNA concentration, 1.5 ng/μl; reaction temperature, 37 °C; reaction time, 1 h. Sequence of the primers: IFF 156: 5'-ATG GCC TGT GCC CAT CTG TGC-3', IFF 157: 5'-GGA CTT CGA GGC AAA GCC AAT-3'.

The cDNA was amplified with the LightCycler® Fast-Start DNA Master^{PLUS} SYBR Green I Kit (Roche Applied Science). The LightCycler apparatus version 1.5 was run with the software version 3.0. The system protocol was as follows: pre-incubation: 10 min at 95 °C. Amplification: (1) denaturation, 5 s at 94 °C, (2) annealing, 5 s at 64 °C, (3) extension, 15 s at 72 °C. Melting curve: denaturation, 0 s at 95 °C; annealing, 15 s at 75 °C; melting, target temperature 95 °C; slope = 0.1 °C/s, continuous. Cooling: 30 s at 40 °C. The concentration of each primer (IFF156 and IFF157) was 0.8 μM.

Five microlitres of diluted RT assay (1 + 1, 1 + 19, 1 + 199, v + v, distilled water) were given to each LC assay, resulting in RNA amounts of 3.75, 0.375 and 0.0375 ng in the LC PCR assay. LC PCR assays were run in duplicate for

each RNA concentration. The crossing line (threshold line) was set to 0.100 in all runs, the fit points method was used to generate the standard curve. The slope, intercept (C_T -value related to 1 ng of RNA) and the regression coefficient were calculated from the linear regression line calculated for three RNA concentrations from the dilution series.

Isoelectric focusing

Extraction of sarcoplasmic proteins and isoelectric focusing (IEF) were performed according to [10], using Servalyt Precotes 3–10 (SERVA, Heidelberg, Germany) of 0.3 mm gel thickness. Protein bands were visualised by staining with Coomassie dye SERVA Violet 17 (SERVA).

“Stains-all” spectra of heat-stable sarcoplasmic proteins were taken as follows: 2 g of fish muscle were homogenized with 6 ml of distilled water using the Ultra-Turrax (Janke & Kunkel, Freiburg, Germany). Non-dissolved material was precipitated by centrifugation, and the supernatant was dialysed three times against 2 l of 10 mM Tris–HCl pH 7.0 at 8 °C using the dialysis tubing Spectra/Por 1 (molecular weight cut-off 6–8 kDa; SERVA, Heidelberg) as described previously [11]. The dialysed solutions were heated at 70 °C for 15 min, and coagulated protein was removed by centrifugation. The protein content of the supernatant was about 0.9–1.5 mg/ml as determined by the biuret method [12].

Staining solution was prepared by mixing 1 ml of 0.11% (w/v) of “stains-all” (4,5,4',5'-dibenzo-3,3'-diethyl-9-methylthiacarbocyanine bromide; SERVA) in formamide with 99 ml 10 mM Tris–HCl pH 8.8. The solution was kept in a brown bottle for up to 8 h at ambient temperature.

Plastic cuvettes were filled with 2.7 ml of staining solution and 0.3 ml of protein solution and the mixture was incubated for 30 min in the dark. Absorbance spectra were measured (Cary 50 Bio, Varian) in the range of 400–700 nm. For comparison, spectra of bovine serum albumin solution (0.1 mg/ml) and of a blank containing Tris buffer instead of protein solution were measured.

Results and discussion

Nucleic acid content of salmon muscle

The amount of DNA extracted per mass unit of light muscle was higher for transgenic salmon than for the non-transgenics (Table 2). This may be explained by hyperplasia, the increase in muscle fibre number, observed for transgenic coho salmon [6]. In rainbow trout (*Oncorhynchus mykiss*) DNA concentration of muscle was positively correlated to fibre density [13]. A similar trend was detected for total RNA (Table 2), but due to the small

Table 2 Amount of DNA and RNA extracted from salmon muscle

Sample code	DNA (µg/g muscle)	RNA (µg/g muscle)
Transgenic		
Fish 2, 2004 T/IV	97	718
Fish 3, 2004 T/IV	66	108
Fish 4, 2004 T/IV	165	483
Fish 5, 2004 T/IV	159	498
Fish 6, 2004 T/IV	83	466
Mean value, SD _{n-1}	114 (45.2)	455 (219)
Non-transgenic		
Fish 13, 2003 T/V	68	215
Fish 14, 2003 T/V	66	392
Fish 15, 2003 T/V	22	459
Fish 17, 2003 T/V	94	202
Fish 18, 2003 T/V	55	76
Mean value, SD _{n-1}	61 (26)	269 (155)
Transgenic, ration-fed		
Fish 26, 2003 T/VI	75	413
Fish 27, 2003 T/VI	ND	419
Fish 28, 2003 T/VI	84	194
Fish 30, 2003 T/VI	90	111
Fish 31, 2003 T/VI	135	551
Mean value, SD _{n-1}	96 (27)	337 (180)

ND not determined

sample size (10–20 mg) a large variation of extractable RNA was found within each group of fish (Table 2).

Species identification

Testing the samples for authenticity by PCR-SSCP analysis of growth hormone amplicon [9] gave the ssDNA pattern expected for coho salmon in case of non-transgenic fish. All specimens of genetically modified coho salmon presented a different pattern (Fig. 1) allowing the differentiation between transgenic and original coho salmon, and verifying their transgenic genotype. This result is explained by the binding of primers IFF 156/IFF157 to respective endogenous growth hormone gene of coho salmon as well as to the introduced growth hormone gene sequence from sockeye salmon (*O. nerka*).

Measurement of parvalbumin mRNA

To compare the formation of parvalbumin in transgenic and non-modified coho salmon, gene expression was followed by RT-PCR without using a housekeeping gene. Recently, the concept of housekeeping genes for normalisation of results has been questioned [14, 15], because most genes are regulated to some extent by internal or external signals. For example, GAPDH and beta-actin, which are often used

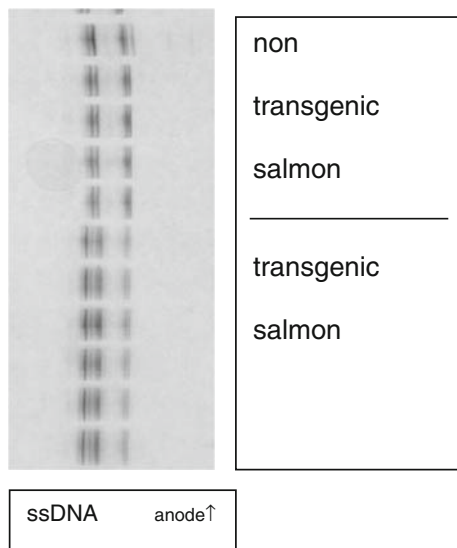


Fig. 1 Differential SSCP patterns of the amplicon of a growth hormone sequence for non-transgenic and transgenic salmon

as housekeeping genes, were found to be up-regulated, respectively, down-regulated, in Nile tilapia (*Oreochromis niloticus*) transferred from freshwater to saltwater [16]. It was therefore decided to relate the C_T -values for parvalbumin

mRNA to a certain amount (1 ng) of total RNA given to the assay.

The RT-PCR method used gave highly reproducible results, as to be seen from the low standard deviation for C_T -values within each group of fish. Although the efficiency of the reverse transcription reaction was not determined, the efficiency of the LC PCR was 1.91, approaching the optimal value of 2, as calculated from the average slope of calibration line (-3.55 , see Table 3). The C_T -values for transgenic and non-transgenic salmon did not differ very much, the latter group having the lower value, meaning the higher copy number of parvalbumin mRNA. When the differences between C_T -values were transformed to differences in copy numbers, the non-transgenic salmon possessed 1.66-fold, respectively, 2.14-fold enhanced parvalbumin mRNA concentration compared to the transgenic or the transgenic ration-fed fish.

ANOVA analysis indicated that the expression of the parvalbumin β gene studied was not enhanced at the RNA level. Thus, the up-regulation of parvalbumin expression in transgenic coho salmon detected by subtractive hybridization [6] was not corroborated by the present study. This result was confirmed by the analysis of sarcoplasmic proteins described in the following section.

Table 3 Comparison of C_T -values obtained by qRT-PCR of parvalbumin mRNA

Sample	3.75 ng RNA C_T -value	0.375 ng RNA C_T -value	0.0375 ng RNA C_T -value	Intercept ^a C_T -value	Slope	Regression coefficient	Control (–RT) C_T -value
Transgenic							
Fish 2	15.84	19.49	22.73	17.89	–3.44	–0.99	35.14
Fish 3	14.36	17.88	21.52	16.30	–3.58	–1.00	33.04
Fish 4	15.33	19.04	22.62	17.44	–3.64	–1.00	32.39
Fish 5	14.87	18.14	21.27	16.74	–3.21	–1.00	28.12
Fish 6	13.80	17.52	21.29	15.94	–3.75	–1.00	35.50
Mean value SD_{n-1}	14.84 (0.799)	18.41 (0.823)	21.89 (0.728)	16.88 (0.787)	–3.52 (0.209)	–1.00 (0.004)	32.84 (2.953)
Non-transgenic							
Fish 13	14.20	17.97	21.48	16.33	–3.64	–1.00	35.57
Fish 14	13.78	17.65	20.88	15.92	–3.55	–1.00	26.90
Fish 15	13.98	17.17	20.58	15.84	–3.30	–1.00	24.97
Fish 17	13.73	17.19	20.98	15.76	–3.63	–1.00	29.69
Fish 18	14.45	18.37	21.75	16.64	–3.65	–1.00	35.10
Mean value SD_{n-1}	14.03 (0.300)	17.67 (0.515)	21.13 (0.473)	16.10 (0.372)	–3.55 (0.149)	–1.00 (0.000)	30.46 (4.793)
Transgenic ration-fed							
Fish 26	16.02	19.38	22.68	17.97	–3.33	–1.00	32.53
Fish 27	15.65	19.49	22.97	17.81	3.66	–0.99	32.36
Fish 28	14.48	18.62	21.95	16.76	–3.74	–1.00	27.91
Fish 30	14.66	18.12	21.66	16.66	–3.50	–1.00	30.61
Fish 31	15.03	18.76	22.10	17.13	–3.53	–1.00	32.29
Mean value SD_{n-1}	15.17 (0.654)	18.87 (0.566)	22.27 (0.539)	17.27 (0.599)	–3.55 (0.157)	–1.00 (0.004)	31.14 (1.966)

^a C_T value calculated for 1 ng RNA

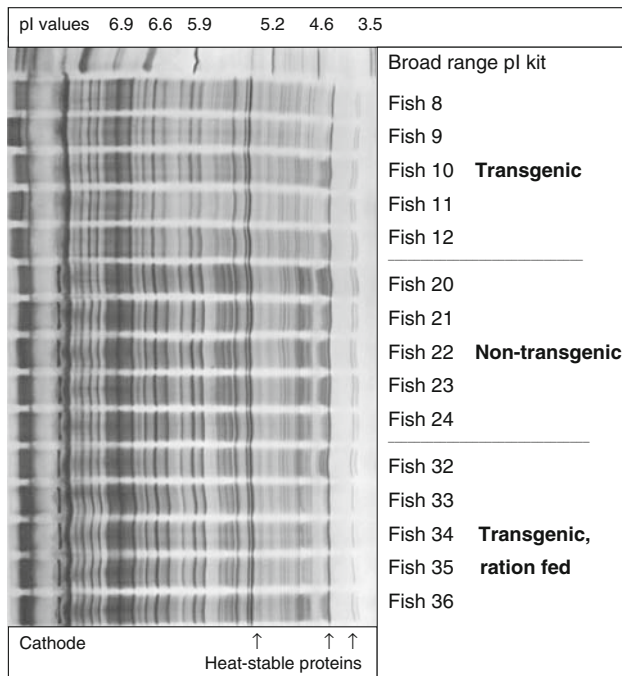


Fig. 2 Protein patterns obtained by IEF of sarcoplasmic proteins

IEF of sarcoplasmic proteins

The protein profiles of transgenic and non-transgenic coho salmon were characterized by the same number and position of protein bands (Fig. 2). As has been described earlier, the protein pattern of salmonid species is determined by strong bands in the neutral and alkaline part of the pH-gradient, as well as by several fainter protein bands of low *pI* value [17]. For the samples of coho salmon analysed in this experiment we have found prominent protein bands in the anodic part of the gel as well (Fig. 2). The *pI* values for these bands were in the range of 5.5 to about 4; these low *pI*-proteins were stable and detectable by IEF when heated at 70 °C for 15 min (data not shown). For white muscle of coho salmon, six acidic proteins had been identified previously by IEF and stains-all staining having *pI* values from 3.81 to 5.06 [17].

As demonstrated in Fig. 2, the strongest bands of proteins, including the presumed parvalbumins, are found for the non-transgenic salmon. Thus, IEF gave no indication of increased parvalbumin concentration due to transgenic modification (Fig. 3).

Staining of heat-stable proteins with stains-all

The selective reactivity of the carbocyanine dye stains-all with acidic, Ca^{2+} -binding protein, has been used to identify, characterize and quantify parvalbumin and related proteins [18, 19]. After enrichment of parvalbumins by heating

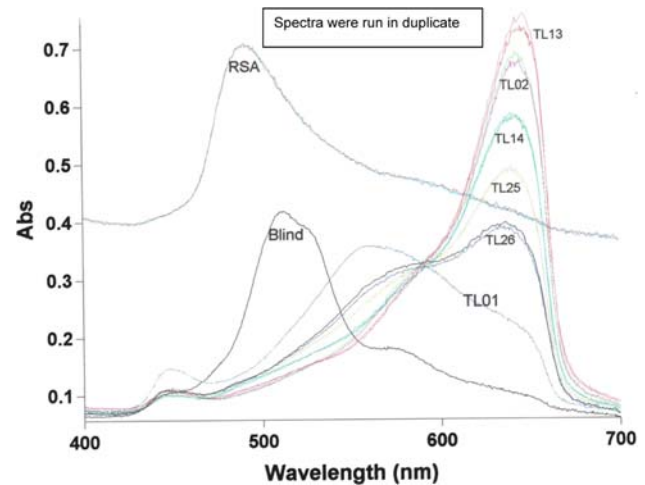


Fig. 3 Stains-all spectra of heat-treated extract of salmon muscle. RSA: bovine serum albumin, blind: without protein; TL01, TL02: transgenic; TL13, TL14, non-transgenic; TL25, TL26: transgenic, ration-fed

extracts of salmon muscle and removal of coagulated protein by centrifugation, the supernatants containing heat-stable proteins were mixed with stains-all solution. The solutions showed a blue to pink colour, indicating the presence of Ca^{2+} -binding proteins. In contrast, the blank, as well as the bovine serum albumin containing solution expressed a red colour.

The absorbance spectra taken from 400 to 700 nm showed maxima around 640 nm, in accordance with the visual appearance of the solutions. The heights of peaks were in the same range for non-transgenic (sample TL13 and TL14) and transgenic (sample TL 02, TL 25 and TL 26) salmon. The spectrum for TL01 was different from all other spectra of salmon extracts, possibly due to insufficient removal of other sarcoplasmic proteins. The spectrum obtained for bovine serum albumin expressed a peak at 494 nm. The results of this staining experiment were consistent with the conclusions drawn from the IEF patterns.

Overall, we found no evidence for enhanced parvalbumin content in GH transgenic salmon muscle. The difference of this result to the enhanced expression of parvalbumin described previously [6] may be due to the smaller size of fish analysed in the former study.

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