The Primary Structures of Growth Hormones of Three Cyprinid Species: Bighead Carp, Silver Carp, and Grass Carp

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The primary structures of growth hormone (GH) of three cyprinid species, bighead carp, silver carp, and grass carp, were determined by a chemical method and/or by molecular cloning. The bighead carp GH was extracted from pituitary tissue by use of an alkaline medium, then successively purified by gel filtration, hydrophobic interaction column chromatography, and reverse-phase high-pressure liquid chromatography. The primary structure of bighead carp GH determined chemically is identical to that deduced from the cDNA nucleotide sequence. By molecular cloning, the primary structures of silver carp and grass carp GHs were also determined. The GHs of these three cyprinid species all contain 188 amino acid residues and their sequences are identical. When four of the five cysteines of cyprinid GHs were arranged to match the same positions of cysteines of other vertebrate GHs, a maximally matched alignment was achieved. Among fishes, GHs are relatively conserved within the same order (82 to 100% identity) but they are more diversified between orders (49 to 68% identity). In further comparison, fish GHs are even more different from tetrapod GHs (37 to 58% identity). Although the primary structures of vertebrate GHs are relatively variable, four homologous sequences, notably one located at the C-terminal, are found. © 1992 Academic Press, Inc.

Growth hormone (GH), a polypeptide hormone secreted from pituitary gland, is required for normal growth and development. GH, together with prolactin and chorionic somatomammotropin, form a family of peptide hormones of similar structure and function. It has been suggested that they have evolved from a common ancestral gene by duplication (Nicoll *et al.*, 1986). The primary structures of GH from several species of vertebrates have been determined, including mammals (Graf and Li, 1974; Martial *et al.*, 1979; Barta *et al.*, 1981; Yamano *et al.*, 1988), birds (Souza *et al.*, 1984; Chen *et al.*, 1988), reptiles (Yasuda *et al.*, 1989), amphibians (Pan and Chang, 1988), and fish (Agellon and Chen, 1986; Nicoll *et al.*, 1987; Yamaguchi *et al.*, 1987, 1989; Noso *et al.*, 1988; Sato *et al.*, 1988; Watahiki *et al.*, 1988; Ho *et al.*, 1989; Koren *et al.*, 1989; Rentier-Delrue *et al.*,

1989; Liu *et al.*, 1991). The available information indicates that fish GHs are very diversified.

In order to obtain more information about the molecular evolution of GH, we have determined the primary structures of GHs of three species of Cyprinidae, bighead carp (*Hypophthalmichthys nobilis*), silver carp (*H. molitrix*), and grass carp (*Ctenopharyngodon idellus*) by a chemical method and/or by cDNA cloning technique. Their nucleotide sequences and amino acid sequences are reported here and their amino acid sequences are compared to the GHs of other species of fish and tetrapods.

MATERIALS AND METHODS

Materials. The enzymes and high-pressure liquid chromatography (HPLC) products were purchased from the following sources: lysyl endopeptidase from

Wako Pure Chemical Industry Ltd., Osaka, Japan: sequencing-grade trypsin from Boehringer, Mannheim, Germany; TSK-ODS-120T column (4.6×250 mm) and TSK Phenyl 5PW column (7.5×100 mm) from Toyo Soda Mfg. Ltd., Tokyo, Japan; Nucleosil C₁₈ (4.6×250 mm) and Synchronapak C₄ column (4.6×250 mm) were self-packed. All other reagents and solvents were of the highest grade available commercially.

Isolation and purification of growth hormone. Bighead carp pituitary glands were collected in ice-water, ruptured by brief sonication, freeze-dried, and stored at -20° until use. To isolate GH, pituitary powder (0.25 g) was suspended in 10 ml of 0.02 M Tris-HCl buffer (pH 9.0) containing 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 1 mM ethylenediamine tetraacetic acid, and 5 mM sodium iodoacetate. After gentle stirring at 4° for 3 hr, supernatant was obtained by centrifugation (18,000g, 30 min at 4°). This is the crude extract of GH.

To purify GH, the crude extract was applied to a column of superfine Sephadex G-75 (1.5×110 cm) equilibrated with 0.05 M ammonium bicarbonate, pH 9.0, and eluted with the same solution containing the above-mentioned protease inhibitors at a flow rate of 6.0 ml/hr at room temperature. The GH-containing fraction was freeze-dried, dissolved in buffer A (10 mM sodium phosphate buffer, pH 7.0, containing 1 M ammonium sulfate), and then subjected to hydrophobic interaction column chromatography in tandem columns of TSK phenyl 5 PW equilibrated with buffer A. The column was first eluted at a flow rate of 1 ml/min with buffer A for 15 min, then eluted with a linear gradient of buffer A to buffer B (10 mM sodium phosphate buffer, pH 7.0) for 60 min, and finally eluted with buffer B for 15 min. The GH-containing peak was collected and purified by reverse-phase (rp) HPLC, first using the Synchronapak C₄ column and then the TSK ODS 120T column. The Synchronapak C₄ column was equilibrated with a mixture of 80% solvent A (0.07% trifluoroacetic acid, TFA) and 20% solvent B (0.07% TFA in acetonitrile) and the sample was eluted with a linear gradient of solvent B which reached from 20 to 70% within 60 min at a flow rate of 1 ml/min. For operation of the TSK ODS 120T column, it was equilibrated with a mixture of 65% solvent A and 35% solvent B and the sample was eluted with a linear gradient of solvent B which reached from 35 to 65% within 45 min at a flow rate of 1 ml/min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor GH which appears as a band with molecular weight (MW) of 20,000.

Sequence determination. Reduction and alkylation of GH and desalting of reduced carboxymethylated (RCM)-GH by HPLC were performed as described previously (Liu *et al.*, 1989). Cleavage of RCM-GH by lysyl endopeptidase was carried out in 50 mM Tris-HCl buffer (pH 9.0) in 4 M guanidine HCl at 37° for 5

hr. A part of lysyl endopeptidase digest was dissolved in 0.3 ml of 50 mM Tris-HCl buffer (pH 8.0) and further treated with trypsin at 37° for 18 hr. The resulting peptides were separated on a Nucleosil C₁₈ column. For operation, the column was equilibrated with a mixture of 90% solvent A and 10% solvent B and the sample was eluted with a linear gradient of solvent B which reached from 10 to 60% within 90 min at a flow rate of 1 ml/min. Microscale amino acid analysis was achieved by a method described previously (Chang and Liu, 1988). Amino acid sequence was determined by means of a pulsed-liquid-type Model 477A sequenator (Applied Biosystems, Foster City, CA) in combination with an on-line phenylthiohydantoin amino acid analyzer.

Construction of cDNA library, screening, and sequencing of GH cDNA. The methods used to construct pituitary cDNA libraries of bighead carp, silver carp, and grass carp were essentially the same as previously described (Chang *et al.*, 1990). Briefly, the pituitary polyadenylated mRNAs were prepared by the guanidinium/CsCl method followed by oligo(dT) cellulose column chromatography. Double-stranded cDNA was synthesized, ligated with EcoRI linker, and then cloned into pUC 19. Carp GH (cGH) cDNA was used as probe to screen the clones containing the cDNA encoding GH of bighead carp (bcGH), silver carp (scGH), and grass carp (gcGH).

The cDNAs encoding bcGH, scGH, and gcGH were cleaved with *Hinc*II for subcloning. The nucleotide sequences were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

RESULTS

Purification of Bighead Carp GH

By Sephadex G-75 gel filtration, 43 mg of GH containing fraction was obtained from 250 mg of pituitary powder (data not shown). The GH fraction from gel filtration was further fractionated by hydrophobic interaction column chromatography. The GH-corresponding peak accounted for 33% of the applied sample (data not shown). Final purification was accomplished by two rpHPLC (Fig. 1). At this stage, GH became more or less insoluble so that the final yield could not be assessed. The purity of GH was ascertained by SDS-PAGE which reveals one major band of MW 20,000 and one minor band of MW 40,000 (Fig. 2).

FIG. 1. Separation of bighead carp GH by HPLC. (a) About 1.5-mg sample from hydrophobic interaction column chromatography was injected onto a Synchropak C₄ column. (b) Chromatography of GH fraction from (a) on a TSK ODS 120T column.

Sequencing of Peptide Fragments

The peptide map of lysyl endopeptide digestion is presented in Fig. 3. Fourteen prominent peaks were separated. Among them, peaks LE-1, LE-7, LE-9, and LE-13 produced unique sequence while peaks LE-4 and LE-8 gave two different sequences. For LE-4, the 2 residues from each sequencing step had a peak area ratio of 11:1. Therefore, such difference was used to distinguish the sequences of LE-4-1 and LE-4-2 which began from positions 166 (LE-4-1) and 169 (LE-4-2), respectively. However, LE-8 gave two sequences in about the same intensity. One of the 2 residues of each sequencing step was identical to the residue determined from the N-terminus of intact GH. The peptide with the same sequence of the first 23 N-terminal residues was denoted as LE-8-1. The sequence of other peptide, LE-8-2, was then determined by subtracting the residues of LE-8-1 from LE-8. Other missing sequences were complemented by peptides resulting from double digestion of lysyl en-

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of bighead carp GH. The sample was dissolved in Tris-HCl buffer, pH 6.8, containing 1.6% SDS and 0.1% 2-mercaptoethanol and heated at 100° for 10 min, then loaded to 10% gel. Left lane, protein markers (from top to bottom) with molecular weights 94,000, 67,000, 43,000, 30,000, 20,100, and 14,400; right lane, bighead carp GH.

FIG. 3. Chromatography of lysyl endopeptidase (LE) digestion of bighead carp GH on an Nucleosil C₁₈ column. One milligram was applied.

dopeptidase and trypsin as represented by LE-T-7, LE-T-15, and LE-T-23. All the sequences of the above peptides thus determined were identical to those deduced from

the nucleotide sequence of cDNA encoding bcGH (see below); therefore the complete sequence of bcGH was aligned and shown in Fig. 4.

FIG. 4. Primary sequence of bighead carp GH aligned from peptides of lysyl endopeptidase (LE) and lysyl endopeptidase/trypsin (LE-T) digestion. Sequences in parentheses are derived from amino acid composition.

*Isolation and Nucleotide Sequencing
of cDNA Encoding bcGH, scGH,
and gcGH*

There were 9, 5, and 13 positive clones obtained from 313, 305, and 295 clones of bighead carp, silver carp, and grass carp pituitary cDNA libraries, respectively. The cDNA of the largest size from each species was subjected to nucleotide sequencing.

The cDNAs encoding bcGH, scGH, and

gcGH were in the range of 1158 to 1171 base pairs (bp) in length. Their nucleotide sequences are almost identical, only differing in a few bases. Consequently, only the sequence of bcGH cDNA was shown (Fig. 5). It contained a 22-bp 5'-noncoding region, a 630-bp open reading frame (ORF), and a 500-bp 3'-untranslated region. The ORF encodes a polypeptide of 210 amino acid residues which includes a leader peptide of 22 residues and a growth hormone of 188

GTAAGAATCTACCCTGATCGAA								ATG Met -22	GCT Ala -20	AGA Arg -20	GCA Ala	TTA Leu	GTG Val	CTG Leu	TTG Leu	TCG Ser	GTG Val					
GTG Val	CTG Leu	GTT Val -10	AGT Ser	TTG Leu	TTG Leu	GTG Val	AAC Asn	CAG Gln	GGG Gly	ACG Thr	GCC Ala -1	TCA Ser 1	GAG Glu	AAC Asn	CAG Gln	CGG Arg	CTC Leu					
TTC Phe	AAC Asn	AAC Asn	GCA Ala 10	GTC Val	ATC Ile	CGT Arg	GTT Val	CAA Gln	CAC His	CTG Leu	CAC His	CAG Gln	CTG Leu 20	GCT Ala	GCA Ala	AAA Lys	ATG Met					
ATT Ile	AAC Asn	GAC Asp	TTT Phe	GAG Glu	GAC Asp 30	AAC Asn	CTG Leu	TTG Leu	CCT Pro	GAG Glu	GAA Glu	CGC Arg	AGA Arg	CAG Gln	CTG Leu 40	AGT Ser	AAA Lys					
ATC Ile	TTT Phe	CCT Pro	CTG Leu	TCT Ser	TTC Phe	TGC Cys	AAC Asn 50	TCT Ser	GAC Asp	TCC Ser	ATT ile	GAG Glu	GCG Ala	CCC Pro	ACT Thr	GGA Gly	AAA Lys 60					
GAT Asp	GAA Glu	ACG Thr	CAG Gln	AAG Lys	AGC Ser	TCT Ser	ATG Met	TTG Leu	AAG Lys 70	CTC Leu	CTT Leu	CGC Arg	ATC Ile	TCT Ser	TTC Phe	CGC Arg	CTC Leu					
ATT Ile	GAG Glu	TCC Ser	TGG Trp	GAG Glu	TTC Phe	CCC Pro	AGC Ser	CAG Gln	ACC Thr	CTG Leu	AGC Ser	GGA Gly 90	GCC Ala	GTC Val	TCA Ser	AAC Asn	AGC Ser					
TTG Leu	ACC Thr	GTC Val	GGT Gly 100	AAC Asn	CCC Pro	AAC Asn	CAG Gln	ATC Ile	ACT Thr	GAG Glu	AAG Lys	CTG Leu 110	GCC Ala 110	GAC Asp	TTG Leu	AAA Lys	GTG Val					
GGC Gly	ATC Ile	AGC Ser	GTG Val	CTC Leu	ATC Ile 120	AAG Lys	GGA Gly	TGT Cys	CTG Leu	GAT Asp	GGT Gly	CAA Gln	CCA Pro	AAC Asn	ATG Met 130	GAT Asp	GAT Asp					
AAC Asn	GAC Asp	TCC Ser	CTG Leu	CCG Pro	CTG Leu	CCT Pro	TTT Phe 140	GAG Glu	GAT Asp	TTC Phe	TAC Tyr	TTG Leu	ACC Thr	ATG Met	GGG Gly	GAG Glu	AGC Ser 150					
AGC Ser	CTC Leu	AGA Arg	GAG Glu	AGC Ser	TTT Phe	CGT Arg	CTT Leu	CTG Leu	GCT Ala 160	TGC Cys	TTC Phe	AAG Lys	AAG Lys	GAC Asp	ATG Met	CAC His	AAG Lys					
GTG Val	GAA Glu	ACT Thr	TAC Tyr	CTA Leu	AGG Arg	GTT Val	GCA Ala	AAT Asn	TGC Cys	AGG Arg 180	AGA Arg	TCC Ser	CTG Leu	GAT Asp	TCA Ser	AAC Asn	TGC Cys					
ACC Thr																		CTG Leu	TAG ***	AGGGCGCCAATGTATTGCTAGCCAAAGCCTGTGACACACTTTGCTGCAAAATCTAAACCC		
AGTTTAAAGTCCTCAAATCTCCTAATATAATTATTATCTGGTTTTATATATGCAGGAAATGTCAACCAGGC																						
ATGGCTAGGTCTGTTCTCTAGCTCCCTCCCATATCTAAACCTACCTAACACTATTGTATTTATTCTTCTC																						
ATTGGGGAGTGCTCGTAAATTAAGACATTAAGATCTGATTTAACATTTTCACAGTGGTGCTAAGCAATTTA																						
TGGCGCTATATTTTCAAATGTGCCCAATCGCTTTGACTCTAGTATTTTATGGCTCCAAAATTGGCTAAAG																						
ATGCCTTTTGTGCAAACTGTCATTTGGATGGATGGGTTCACTCCCAACCAAGTGTATGAATGTAAACATTT																						
GTCTGTCTGATTATGTCCATATTATTAGCTCATGCTGTTCTCTTGAAGCTGTGTCTTTATCCATTAAAG																						
TTCTAAACTGG-PolyA																						

FIG. 5. Nucleotide sequence and deduced amino acid sequence of cDNA encoding bighead carp GH.

amino acids. A polyadenylation signal (ATTAAG) is present in the 3'-untranslated region near the polyadenylate tract.

The scGH and gcGH cDNA had the same structure as bcGH cDNA, including a 5'-noncoding region of 28 to 23 bp, an ORF of 630 bp, and a 3'-untranslated region of 512 to 518 bp. Their ORF also encoded a leader peptide of 22 residues and a GH of 188 residues. The sequences of scGH and gcGH thus deduced are completely identical to that of bcGH.

DISCUSSION

In this study, bcGH was monitored in SDS-polyacrylamide gels by assuming that the MW of bcGH is 20,000. No bioassay was performed. However, there is reason to assume that the fraction thus purified is bcGH because its amino acid sequence is homologous to the known GH sequences of other species (see below). After the complete sequence had been determined, the MW of bcGH was calculated to be 21,101,

	10	20	30	40	50
Bighead carp	SENQRLFNNAVIRVQHLHQLAAKMINDFEDNLLPEERRQLSKIFPLSFCN				
Salmon	I-----I--S-----L--Q--F--DGT---D-----N--L-D---				
Tilapia	TDS---SI--N--T---L--QRLFS---SS-QT--Q---N---LQD---				
Tuna	TDS---SI--S-----L--QRLFS---SS-QT--Q---N---LQD---				
Pike eel	VEPISLYN--TS--S-A---T---TIYKE--RSIP--AH-----TS--AG-Y				
Blue shark	YPLPLSD--AK--H-A---V--ETTK---RKYI---Q-HSH-SS-SA--Q				
Bullfrog	FPQMSLSN--T---A-----MV-DTYR-Y-RTYI--DQ-FKQTLISVY-Y				
Sea turtle	AFPAMPLSS--A--L-----L---DTYKEF--RTYI--EQ-HSN-ISQSAS-Y				
Chicken	AFPAMSLSG--A--L-A---L---ETYKEF--TYI--DQ-YTN-NSQAA--Y				
Bovine	TFPAMPLSN--A--L-A-----DTFKEF--TYI--GQ-YSIQNTQVA--F				
	60	70	80	90	100
Bighead carp	SDSIEAPTGKDET QKSSMLKLLRISFRLIE SWEFPSQTLSGAVSNSLTVG				
Salmon	---VS-VD-H---V---H-----Y-----II---M-R				
Tilapia	--Y-IS-ID-H---R--V---S--YG-V- ---RS---GS--R				
Tuna	--Y-IS-ID-H---R--V---S--Y--V- ---RS---GAP R				
Pike eel	---LPT-----EK-DGY-----SA-Q --VY-LK--D-F---MF-				
Blue shark	-ET-P-----EDA -QR-DEL--YSLL --Q --LN-I-N--AFRTSDR -Y				
Bullfrog	-T-P---D--N-H---D ID---F-LT-TLLQ--MT-I-IVNRVFN-Q VF-				
Sea turtle	-ET-P---D-Q---D- E---F-LI-Q ---N-V-F--RVFT-S-VF-				
Chicken	-ET-P---DAQ---D- E---F-LV--Q --LT-V-Y--KVFT-N-VF-				
Bovine	-ET-P---N-AQ---DL E-----LL--Q --LG-L-F--RVFT---VF-				
	110	120	130	140	150
Bighead carp	NPNQITEKLADLVKGVISVLIKGCLDGQPNMDDNDSLPLPFEDFY LTMGES				
Salmon	-A---S---S-----NL--T-SQ--VLSL-----QQ--PTGN-YQNLG-DG				
Tilapia	- --SPP-SE--P--LL--RANQ-EAE-YP-T-THQH-PYGN-YQNLG-NE				
Tuna	- --SP--SE--T--HL--RANQ--AEMFA-SSA-Q --PYGN-YQSLGADE				
Pike eel	TSDG-FD--E--NK--NE-M-VVG--GIY-IEDLRKL- YENFDVHLRND				
Blue shark	D--R---E--FA-M-TLE--GSSQGFVW -KFSY-R-D GNLS-E				
Bullfrog	-IDRVYDR-R--E -LHI--RELD--NVRNYGVLTFTYDKF-VN -RS-E				
Sea turtle	TSDRVY---K--EE--QA-MRELE--SLRGFVLRPTYDKF-IN -RNED				
Chicken	TSDRVF---K--EE--QA-MRELE-RS-RGPQLLRPTYDKF-IH -RNED				
Bovine	TSDRVY---K--EE--LA-MREVE--T-RAGQILKQTYDKF-TN M RSDD				
	160	170	180	188	
Bighead carp	SLRESFRLACFKKMDHKVETYLVRVANCRRSLDSNCTL				
Salmon	NV-RNVE-----T--K--K--EA---				
Tilapia	L--RNVE-----T--K--L-PE---				
Tuna	S--R-YE-----T--K--L-PEA---				
Pike eel	G-MKNYG-----K-TK--FVE---				
Blue shark	A-MKNYG-----K-M--K--FAE---V				
Bullfrog	GRAKNYG-----K-MK--FVE---F				
Sea turtle	A-LKNYG--S-----L-----KLMK---FGE---I				
Chicken	A-LKNYG--S-----L-----K-MK---FGE---I				
Bovine	A-LKNYG--S--R--L--T-----MK---FGEAS--AF				

FIG. 6. Primary sequences of GH of representative species. Dashes indicate residues identical to those of bighead carp GH. References used: salmon, Nicoll *et al.*, 1987; tilapia, Rentier-Delrue *et al.*, 1989; tuna, Sato *et al.*, 1988; pike eel, Liu *et al.*, 1991; blue shark, Yamaguchi *et al.*, 1989; bullfrog, Pan and Chang, 1988; sea turtle, Yasuda *et al.*, 1989; chicken, Souza *et al.*, 1984; bovine, Graf and Li, 1974.

which is very close to the MW of the major band of bcGH determined by SDS-PAGE, and also to those of GHs of other fish species such as salmon (Nicoll *et al.*, 1987) and bonito (Noso *et al.*, 1988). The SDS-PAGE shown in Fig. 2 also revealed that the purified bcGH contained a minor band with MW 40,000. Probably, this is the dimer of bcGH. The dimeric form of human GH has been reported (Lewis *et al.*, 1977).

Protein sequencing by the chemical method requires a large amount of sample and is very time-consuming. We combined the techniques of chemical method and molecular cloning to determine the sequence of bcGH. In the present study, only lysyl endopeptidase and trypsin were used; therefore no overlapping sequences were obtained. The sequencing data from the above peptides were completely fitted with those deduced from bcGH cDNA; therefore these nonoverlapping sequences of bcGH could be aligned. Apparently, the complement of the sequencing data determined by different methods gives a more reliable result.

The primary structures of bcGH, scGH, and gcGH are identical. It is noted that the sequence of gcGH cDNA determined by us was different from that determined by Ho *et al.* (1989), i.e., 14 nucleotide (nt) substitutions, 1 nt deletion, and 6 nt insertions. The amino acid sequence of gcGH deduced by us had two residues different from those

deduced by Ho's group, i.e., Ser⁵¹ to Cys⁵¹ and Ala⁹² to Glu⁹². Whether they are two different forms of gcGH is currently unknown. However, it should be pointed out that serine is the 51st residue of GHs of all species thus far investigated (Fig. 6).

The GHs of four species of Cyprinidae, bighead carp, silver carp, grass carp, and common carp, have five cysteine residues, as opposed to four residues found in other species of fish and tetrapods. Except Cys¹²³, the other four cysteines of cyprinid GHs could be aligned at positions corresponding to those of the GHs of other species. By using these four cysteines as a skeleton and inserting or deleting certain residues, the maximally matched sequences of GHs of various species could be arranged (Fig. 6) and the results of homology analysis are presented in Table 1.

The homology between the GHs of four cyprinid species is very high, up to 94% (Koren *et al.*, 1989). Such a high degree of GH homology is also observed among various species within the same order of fish, regardless of whether they belong to the same or different families. Examples are 100% identity between salmon and trout (Salmonidae, Nicoll *et al.*, 1987; Agellon and Chen, 1986) of Salmoniformes; 97% identity between eel (Anguillidae, Yamaguchi *et al.*, 1987) and pike eel (Muraenesocidae, Liu *et al.*, 1991) of Anguilliformes; and 83 to 91% identity among tilapia (Ci-

TABLE 1
HOMOLGY ANALYSIS OF GROWTH HORMONE

Species	1	2	3	4	5	6	7	8	9	10
Bighead carp (1)	100									
Salmon (2)	68	100								
Tilapia (3)	56	67	100							
Tuna (4)	54	66	89	100						
Pike eel (5)	52	48	45	46	100					
Shark (6)	49	36	47	42	45	100				
Frog (7)	44	42	43	40	52	53	100			
Turtle (8)	45	40	45	40	58	68	64	100		
Chicken (9)	45	40	46	38	57	60	63	89	100	
Bovine (10)	43	37	38	37	53	61	56	76	78	100

chilidae, Rentier-Delrue *et al.*, 1989), tuna (Scombridae, Sato *et al.*, 1988), bonito (Scombridae, Noso *et al.*, 1988), and yellow tail (Carangidae, Watahiki *et al.*, 1988) of Perciformes. On the other hand, the GH homology between the species of different orders is decreased to the range of 48 to 68% (Table 1). Apparently, the primary structures of fish GHs are relatively conserved within the same order but become diversified between different orders.

The phylogenetic relationship of the primary structures of GHs among vertebrates is presented in Table 1. As expected, GH from one species of fish is more homologous to the GH of other species of fish than it is to the GHs of tetrapods. Although each vertebrate species has its unique GH, all vertebrate GHs share some common features: (1) 4 cysteine residues (except 5 in Cyprinidae) which can form two disulfide bridges; (2) one conserved tryptophan residue at 82nd position (except 2 residues in blue shark); and (3) four homologous sequences, i.e., residue 6 to 22, 60 to 82, 110 to 119 and notably 158 to 180. In addition, a potential glycosylation site (Asn-Cys-Thr) is found in the C-terminal part of GHs of all vertebrates except mammals. Whether the cyprinid GHs are glycosylated as reported in chum salmon (Wagner *et al.*, 1985) is unknown at present.

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