Journal of Chromatography, 547 (1991) 131–144
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 260

Characterization of recombinant eel growth hormone

SEIJI SUGIMOTO*, KAZUO YAMAGUCHI and YOSHIHARU YOKOO
Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3–6–6 Asahimachi, Machida, Tokyo 194 (Japan)
(Received February 1st, 1991)

ABSTRACT

The characterization of purified recombinant eel growth hormone (rEGH) is described. N-Terminal sequence analysis, amino acid composition analysis, and tryptic mapping confirmed that the primary structure of rEGH was identical with that of its natural counterpart except for an additional Met at the N-terminus. Peptide mapping also revealed that rEGH had two disulphide bonds, Cys49–Cys160 and Cys177–Cys185, as in mammalian growth hormones (GHs). Recombinant EGH was classified as an α-helix-rich protein, similar to mammalian GHs, from the circular dichroism spectrum. Recombinant EGH was immunochemically identical with pituitary-derived EGH. Gel filtration chromatography and light-scattering analysis indicated that rEGH exists as the monomer, as does pituitary-derived EGH.

INTRODUCTION

Several vertebrate growth hormones (GHs) have been isolated [1–6]. Some of these GHs have been expressed by recombinant techniques in *Escherichia coli* (*E. coli*) [1,3,4,7–9]. Human GH has been applied in dwarfism and bovine GH is being developed to stimulate lactation in dairy cows [10–12]. These applications followed from the identification of the three-dimensional structure and biological activity of recombinant GHs with those of pituitary-derived GHs [13]. Teleost GHs are also expected to be useful in promoting growth in aquaculture. We have produced purified recombinant salmon GH (unpublished results) and recombinant eel GH (rEGH) [14–15]. However, the characterization of recombinant teleost GHs has not been reported.

Two forms of eel GH (EGH I and II) were isolated from eel pituitaries and their primary structures were determined by Kishida *et al.* [16] and Yamaguchi *et al.* [17]; EGH II lacks three amino acids present at the N-terminus in EGH I. Molecular cloning of EGH cDNA and its expression in *E. coli* were performed by Saito *et al.* [4].

We have reported a preparation of rEGH, in which expressed in *E. coli* as inclusion bodies was refolded and purified [14]. However, we found minor analogues (deamidated, oxidated and/or formylated rEGH) in the purified rEGH by polyacrylamide gel electrophoresis and reversed-phase high performance liquid chromatography (RP-HPLC) [18]. Subsequently we investigated the causes of these modifications. Recently we established a procedure for the large-scale preparation of rEGH free

0021-9673/91/$03.50

from these analogues [15]. This was the first report of the purification of recombinant teleost GH.

In this paper, we describe the biochemical and biophysical characterization of rEGH.

Natural human, bovine and ovine GHs have already been characterized by several methods. These investigations showed that these GHs have close similarity not only in their primary structure but also in the location of disulphide bonds, in their secondary structure, etc. Disulphide bonds in these GHs were located at the same position. These GHs were classified as α-helix-rich protein by circular dichroism spectrometry [8,13,19–25].

Molecular weight was also investigated. However, it is not yet clarified whether dimerization of these GHs takes place. Ten years ago, molecular weight measurements of pituitary-derived GHs in neutral pH solution by gel filtration chromatography (GFC), osmotic pressure and sedimentation equilibrium methods revealed that human GH exists as a monomer [20,22] and other mammalian GHs as a dimer [21]. Fernandez and Delfino [24], however, reported that pituitary-derived bovine GH showed the characteristics of a rapid monomer–dimer equilibrium whose dissociation constant was determined as $6.6 \cdot 10^{-6}$ *M* by frontal analysis. This means that the molecular weight of bovine GH in neutral pH solution depends on the concentration of bovine GH. Recently, several investigators purified recombinant bovine GH expressed in *E. coli* and showed that the molecular weight of recombinant bovine GH was identical with that of pituitary-derived bovine GH by GFC [8,13,25]. Langley *et al.* [13] showed that pituitary-derived bovine GH and recombinant bovine GH, whose tertiary structure was identical with those of the natural counterparts by several characterization methods, existed as a monomer (18 kilodalton). However, Wingfield *et al.* [8] showed that both the pituitary-derived and recombinant bovine GHs existed as a dimer (35 kilodalton). Brems *et al.* [25] reported that recombinant bovine GH was not eluted from a TSK-2000 column when the mobile phase contained less than 1.0 *M* guanidine hydrochloride (GuHCl). The GFC results could be baised by the chromatographic process, *e.g.*, by sample dilution or binding of protein to the resin.

In this work, to characterize the quaternary structure of rEGH, we particularly measured the molecular weight of rEGH by several methods and found that it exists as a monomer in neutral pH solution.

EXPERIMENTAL

N-Terminal sequence analysis

N-Terminal sequence analysis was carried out with a Model 470A gasphase sequencer (Applied Biosystems). Recombinant EGH preparation (2300 pmol) was dialysed against 0.1% SDS and the dialysate was applied to the sequencer.

Amino acid composition analysis

Protein and peptides were hydrolysed in 6 *M* hydrochloric acid at 110°C for 22 h. Amino acid analysis was carried out by the Waters Pico Tag method of Bidlingmeyer *et al.* [26]. Amino acid standard solution was purchased from Wako.

Tryptic mapping of rEGH

A 196-μg amount of rEGH in 200 μl of 10 m*M* Tris–HCl (pH 8.0) was digested with trypsin–TPCK (Worthington) for 4 h at 37°C using 1% (w/w) enzyme. The digestion was stopped by acidification with 1 *M* hydrochloric acid to pH 2–3. RP-HPLC was carried out on a TSK gel ODS-120T column (30 × 0.46 cm I.D.) with a Tri-Rotar SR2 HPLC system. Linear gradient elution from 0 to 70% acetonitrile in 0.1% trifluoroacetic acid was performed in 60 min. The column oven temperature was kept at 35°C. The flow-rate was 0.7 ml/min. UV detection was carried out at 220 nm.

S-Carboxymethylation

Each of the peaks of T15 and T39 was dried, reduced in 0.5 ml of 6 *M* GuHCl–1 m*M* EDTA–2 m*M* dithiothreitol–100 m*M* Tris–HCl (pH 8.3) at 37°C for 1 h, and subsequently S-carboxymethylated by adding 5 μl of 700 m*M* iodoacetic acid. RP-HPLC was carried out as described above, except with a linear gradient from 0 to 43.2% acetonitrile in 0.1% trifluoroacetic acid in 80 min.

Circular dichroism (CD)

CD measurements were made at room temperature with a J-500 A spectropolarimeter, (JASCO). The protein concentration in solution was 0.4–0.5 mg/ml. The buffer was 10 m*M* Tris–HCl (pH 8.0).

A cell of path length 0.14 mm was used. The full-scale range was 50 millidegree, the time constant was 1 s and the scan speed was 10 nm/min. The mean residue ellipticities [θ], in degrees cm^2/dmol, were calculated using a mean residue mass of 113.4 dalton for rEGH and 115.9 dalton for pituitary-derived human GH. The secondary structure content was calculated from the mean residue ellipticity at 208 nm by the method of Greenfield and Fasman [27].

Protein concentration determination

Protein concentration was determined using a Bio-Rad Labs. protein assay kit [28] using bovine serum albumin as a standard.

Radioimmunoassay (RIA)

The procedures demployed for the purification of pituitary-derived EGH and the preparation of antisera against the pituitary-derived EGH have been described previously [16]. Iodination of pituitary-derived EGH and RIA using a double antibody method were performed as described by Kishida and Hirano [29].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE and staining with Coomassie Brilliant Blue were carried out essentially as described by Laemmli [30]. Stacking gels contained 4% (w/v) acrylamide and separation gels contained 14% (w/v) acrylamide. The sample buffer contained 2-mercaptoethanol (5%), except for samples referred to as unreduced.

Gel filtration chromatography (GFC)

GFC was carried out on a YMC-Pack Diol-120 column (30 × 0.8 cm I.D.) (YMC) with a Model 302 HPLC system (Gilson). Isocratic elution with 100 m*M*

phosphate buffer–200 m*M* NaCl (pH 7.2) including GuHCl at various concentrations, were performed in 60 min at ambient temperature. The flow-rate was 0.7 ml/min and the injection volume was 100 μl. UV detection was carried out at 280 nm. A molecular weight standard was obtained from Pharmacia. Samples were prepared by appropriate dilutions from stock solutions of 200 m*M* sodium chloride–100 m*M* phosphate buffer (pH 7.2), 8 *M* GuHCl–200 m*M* sodium chloride–100 m*M* phosphate buffer (pH 7.2) and 1 mg/ml rEGH–100 m*M* ammonium sulphate–10 m*M* Tris–HCl buffer (pH 8.0). The final protein concentration was 0.2 mg/ml.

Light scattering

Light-scattering analysis was carried out with a DLS-700 dynamic light-scattering spectrophotometer equpped with helium–neon laser at 632.8 nm (Otsuka Electronics). Recombinant EGH in 100 m*M* ammonium sulphate–10 m*M* Tris–HCl buffer (pH 8.0) was concentrated to 6.5 mg/ml using a YM-10 ultrafiltration membrane (Amicon) and diluted to 1.3, 2.6, and 3.9 mg/ml with the same buffer. Dust-free samples were prepared by filtering each solution through a Millex-GV 0.22-μm filter (Millipore), before analysis. All data were collected at 25°C and a 90° scattering angle. Refractive indeces were measured with an RM-102 refractometer (Otsuka electronics). The molecular weight of rEGH was calculated utilizing Debye's light-scattering equation [31], $KC/R_{90} = 1/M + 2A_2\ C + 3A_3C^2 + \ldots$, where $K = 2\pi^2 n_0^2 (\mathrm{d}n/\mathrm{d}C)^2 / N_A\lambda^4$, R_{90} is the Rayleigh ratio when the angle formed between the directions of the incident and scattered rays is 90°, C is the concentration of protein, M is the molecular weight of the protein, n and n_0 are the refractive indeces of the protein solution and the solvent, respectively, $\mathrm{d}n/\mathrm{d}C$ is the refractive index increment of the protein, N_A is Avogadro's number, λ is wavelength of laser and A_2, A_3, etc., are the second, third and higher virial coefficients, using the software with the DLS-700 dynamic light-scattering spectrophotometer.

RESULTS AND DISCUSSION

N-Terminal sequence analysis

The results of automated N-terminal sequence analysis of the rEGH are shown in Table I. Analysis was carried out up to the tenth residue. The sequencing result matched exactly the known sequence of pituitary-derived EGH II [16], but with an additional Met at the N-terminus. A sequence without the additional Met was not detected.

The design of the rEGH expression plasmid [4] was such that the coding sequence begins with an initiation codon for formyl-Met. Recombinant EGH, expressed in *E. coli* cells as inclusion bodies, consisted of 90% of rEGH with Met at the N-terminus and 10% of rEGH beginning with formyl-Met, which as detected by RP-HPLC [18] and removed in the purification process [15]. The sequencing result indicated that rEGH was unprocessed in terms of removal of the N-terminal Met residue. This observation supports the recent suggestion of Hirel *et al.* [32] that initiating Mets followed by amino acid residues with a side-chain of large radius are not removed in prokaryotes.

The presence of an N-terminal Met did not seem to affect the biological potency in a receptor binding assay [33] of the bacterially synthesized EGH, which is not

TABLE I

N-TERMINAL AMINO ACID SEQUENCE OF RECOMBINANT EGH

Cycle	Residue	Yield (pmol)
1	Met	340
2	Ile	570
3	Ser	40
4	Leu	320
5	Tyr	560
6	Asn	270
7	Leu	310
8	Phe	450
9	Thr	40
10	Ser	20

surprising because a human GH variant present in pituitary human GH preparations at about 5% (fast-human GH) has a blocked N-terminal end, owing to the presence of an acetyl group [34].

TABLE II

AMINO ACID COMPOSITION OF RECOMBINANT EGH

Residue	Theoretical number of residues[a]	Calculated number of residues[b]
Asx	22	20.7
Glx	16	17.1
Ser	16	15.0
Gly	11	11.6
His	5	4.5
Arg	9	8.6
Thr	11	10.8
Ala	10	10.7
Pro	6	5.3
Tyr	9	8.4
Val	9	8.9
Met	5[c]	4.9
Ile	10	11.4
Leu	22	22.2
Phe	8	8.4
Lys	14	13.7
Cys	4	ND[d]
Trp	1	ND

[a] The theoretical number of residues is based on the sequence of Saito *et al.* [4].

[b] The calculated number of residues represents the average of values obtained from three separate hydrolyses.

[c] The theoretical value of Met includes an additional Met at the N-terminus.

[d] ND, not determined.

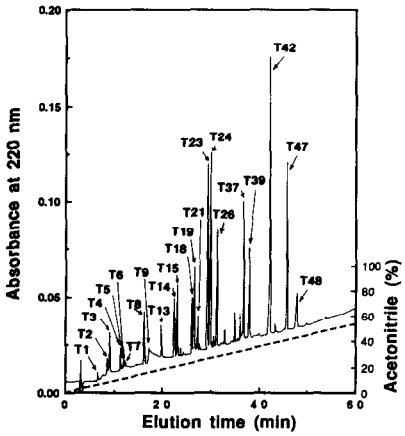


Fig. 1. Tryptic mapping of rEGH by RP-HPLC. A 20-μl volume of the digest was injected.

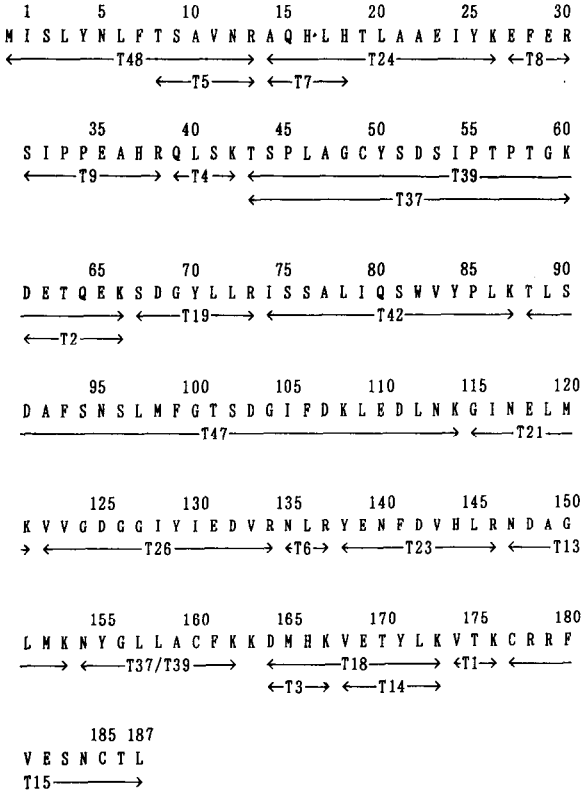


Fig. 2. The deduced amino acid sequence of EGH II cDNA and the alignment of peptides from rEGH. Tryptic peptides derived from rEGH (see Fig. 1 and Table III) are shown with numbered arrows.

Amino acid composition analysis

Results of the amino acid composition analysis of rEGH (Table II) were in good agreement with the theoretical values.

Tryptic mapping of rEGH

A tryptic digest of rEGH was analysed by RP-HPLC (Fig. 1). Correspondence between peaks and fragments and the results of amino acid composition analysis of each peak are summarized in Fig. 2 and Table III, respectively. All fragments of rEGH were detected except Lys163. The results indicated that the primary structure of rEGH was identical with that of pituitary-derived EGH.

Lys163 seemed not to be retained by its hydrophilicity. Peaks T5 and T7 seemed to be produced by contaminated chymotryptic activity in the trypsin preparation. The C-terminal peptide bond of Lys108 was not cleaved. This observation supports the suggestion of Schroeder *et al.* [35] and others [36,37] that an acidic amino acid residue just before and/or after Arg or Lys interrupts cleavage of peptide bonds after these basic residues in trypsin digestions. The C-terminal peptide bond of Arg178 also was not cleaved. This interruption seemed to be caused by the steric hindrance of the disulphide bond between Cys177 and Cys185 (see below). A broad peak, T9, was fractionated into four fractions. The amino acid compositions of each of them were very similar and corresponded to a Ser31–Arg38 fragment (data not shown). This result indicated that a single fragment eluted as a broad peak. This observation supports the suggestion of Gesquiere and Diesis [38] that the peak of a peptide including a Pro–Pro sequence in RP-HPLC is broad or split because of slow *cis–trans* isomerization of Pro–Pro bonds.

The results of N-terminal amino acid sequence analysis, amino acid composition analysis and tryptic mapping revealed that rEGH had the natural primary structure with an additional Met at the N-terminus.

Location of disulphide bonds

Amino acid composition analysis (cysteine and cystine were not detected) of peaks T15 and T39 of tryptic mapping (Fig. 1) revealed that these peaks correspond to a mixture of equal amounts of fragments Thr43–Lys60 and Asn154–Lys162 and to that of fragments Cys177–Arg179 and Phe180–Leu187, respectively. No other peak of which the amino acid composition corresponded to fragments including a cysteine residue was detected.

The rechromatograms of the reduced and carboxymethylated peptides from peaks T15 and T39 are shown in Figs. 3 and 4, respectively. A single peak T15-1 was detected on the rechromatogram of carboxymethylated peak T15. The amino acid composition of peak T15-1 and the difference in the amino acid composition between peaks T15 and T15-1 corresponded to Phe1180–Leu187 and Cys177–Arg179, respectively. Therefore, a disulphide bond between Cys177 and Cys185 is suggested. Fragment Cys177–Arg179 seemed not to be retained by its hydrophilicity. Two peaks, T39-1 and T39-2, were observed for peak T39. The amino acid compositions of the peptides corresponded to those of the fragments Thr43–Lys60 and Asn154–Lys162, respectively. These results indicated that another disulphide bond was formed between Cys149 and Cys160.

Unfortunately, the positions of the disulphide bonds in pituitary-derived EGH

were not confirmed, whereas those of pituitary-derived human and bovine GHs were confirmed at Cys49–Cys160 and Cys177–Cys185 [22,23]. It is assumed, however, that the locations of disulphide bonds are conserved among vertebrate GHs, because the primary structure of vertebrate GHs is conserved well from human to teleost, and especially the position of Cys is conserved at all four sites [17,39]. It is concluded, therefore, that the location of the disulphide bonds in rEGH is the same as that in pituitary-derived EGH.

CD

Fig. 5 shows the far-UV CD spectra of rEGH and pituitary-derived human GH (Sigma). The far-UV CD spectrum of rEGH was very similar to that of pituitary-derived human GH. Both spectra had negative bands at 208 and 219 nm. The far-UV CD spectrum reflects the secondary structure of a protein [40,41]. The spectra classified these GHs as α-helix-rich protein [40,41]. α-Helix contents of rEGH and pituitary-derived human GH were calculated as 45% and 56%, respectively, using Greenfield and Fasman's method [27]. Pituitary-derived vertebrate GHs were classified as

TABLE III

AMINO ACID ANALYSIS OF TRYPTIC PEPTIDE FROM RECOMBINANT EGH

Values represent the average number of residues per molecule obtained from three separate hydrolyses. Numbers in parentheses represent the number of residues determined by the sequence deduced from EGH cDNA [4]. Conditions for trypsin digestion, chromatography and amino acid composition analysis as described under Experimental.

Residue	Fragment no.										
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T13	T14
Asx[a]		1.0(1)	0.8(1)		1.1(1)	1.0(1)				2.0(2)	
Glx[a]		3.0(3)		0.9(1)			1.0(1)	2.1(2)	1.2(1)		1.0(1)
Ser				0.9(1)	1.0(1)				0.9(1)		
Gly										1.0(1)	
His			1.0(1)				1.7(2)		0.8(1)		
Arg					1.0(1)	0.9(1)		1.0(1)	0.9(1)		
Thr	0.9(1)	1.0(1)			1.0(1)						0.8(1)
Ala					1.1(1)		1.0(1)		1.1(1)	1.0(1)	
Pro									1.9(2)		
Tyr											1.0(1)
Val	1.0(1)				1.0(1)						1.0(1)
Met			0.2(1)							0.4(1)	
Ile									1.2(1)	0.9(1)	
Leu				1.1(1)		1.0(1)	1.3(1)				1.1(1)
Phe								1.0(1)			
Lys	1.0(1)	1.0(1)	1.2(1)	1.1(1)						1.0(1)	1.2(1)
Cys											
Trp											
Total	3	6	4	4	6	3	5	4	8	7	6
Position	174–176	61–66	164–167	39–42	8–13	135–137	14–18	27–30	31–38	147–153	168–173

[a] Glx, Asx, acid + amide.
[b] ND, not detected.

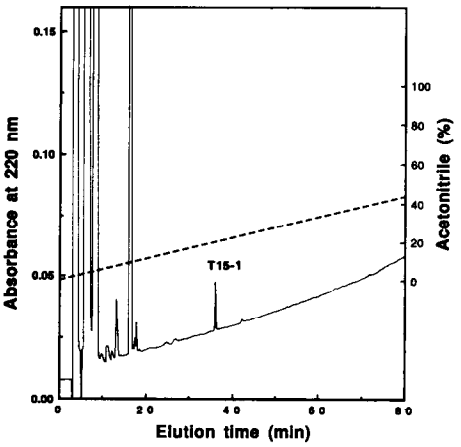
α-helix-rich protein. The α-helix contents of human, bovine, ovine, and salmon GHs were reported to be 50–60%, 50%, 40–45% [20,21] and 48% (unpublished data), respectively. The content for human GH (56%) was in good agreement with the previously reported value of 50–60%. Recombinant EGH was also classified as α-helix-rich protein as well as other vertebrate GHs, and the content was similar to those of bovine, ovine or salmon GH rather than human GH.

RIA

The RIA results showed equivalence between rEGH and pituitary-derived EGH, in terms of recognition by the polyclonal antibody used (data not shown). As antibody–ligand binding is known to be conformation specific rather than sequence specific, correct conformation of rEGH was confirmed by the RIA results. Recently, Hirano [33] has shown that hepatic GH receptor of the eel does not distinguish rEGH from pituitary-derived EGH.

The RIA and receptor assay results further suggested that the three-dimensional structure of rEGH was virtually identical with that of its natural counterpart.

T15	T18	T19	T21	T23	T24	T26	T37	T39	T42	T47	T48
1.0(1)	0.9(1)	1.0(1)	1.0(1)	1.8(2)		2.1(2)	2.9(3)	2.3(2)		5.9(6)	2.2(2)
1.1(1)	1.2(1)		1.2(1)	1.3(1)	2.0(2)	1.4(1)	2.9(3)		1.0(1)	1.3(1)	
1.0(1)		1.1(1)					2.6(3)	2.8(3)	2.5(3)	3.6(4)	1.7(2)
		1.1(1)	0.9(1)			3.0(3)	2.6(3)	3.1(3)		2.0(2)	
	1.0(1)			0.7(1)	1.8(2)						
1.9(2)		1.0(1)		0.8(1)		0.8(1)					0.7(1)
1.0(1)	1.3(1)				1.1(1)		3.9(4)	2.8(3)		2.0(2)	0.7(1)
					3.1(3)		2.1(2)	1.8(2)	0.9(1)	1.1(1)	0.7(1)
							3.2(3)	2.7(3)	0.9(1)		
	0.9(1)	0.9(1)		1.1(1)	1.0(1)	1.0(1)	2.1(2)	2.0(2)	1.0(1)		1.1(1)
1.0(1)	0.9(1)			1.2(1)		2.5(3)			1.2(1)		0.9(1)
			0.8(1)							0.9(1)	1.0(1)
			1.2(1)		1.1(1)	2.3(2)	1.3(1)	1.4(1)	2.4(2)	1.1(1)	1.4(1)
1.1(1)	1.1(1)	1.9(2)	1.0(1)	1.1(1)	2.2(2)		3.4(3)	3.3(3)	2.2(2)	4.2(4)	2.3(2)
1.0(1)				1.1(1)			1.3(1)	1.2(1)		3.2(3)	1.4(1)
	1.9(2)		0.9(1)		0.9(1)		2.6(3)	1.7(2)	0.9(1)	1.7(2)	
ND[b](2)							ND (2)	ND (2)			
									ND (1)		
11	19	7	7	9	13	13	33	27	14	27	14
177–187	164–173	67–73	115–121	138–146	14–26	122–134	43–66	43–60	74–87	88–114	0–13
							154–162	154–162			



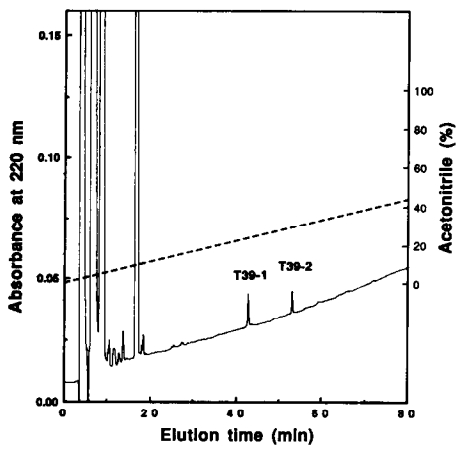
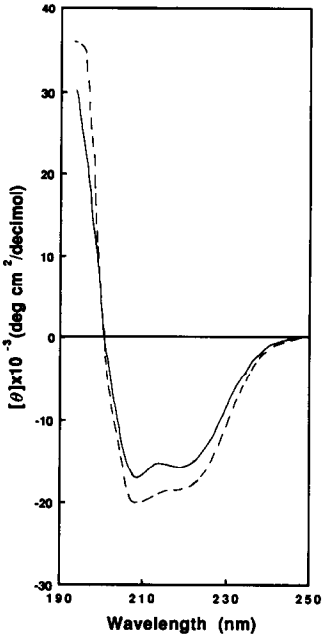


Fig. 3. HPLC separation of carboxymethylated peptides derived from peak T15, tryptic peptide of rEGH. A 450-μl volume of carboxymethylated peptide solution was injected.

Fig. 4. HPLC separation of carboxymethylated peptides derived from peak T39, tryptic peptide of rEGH. A 450-μl volume of carboxymethylated peptide solution was injected.



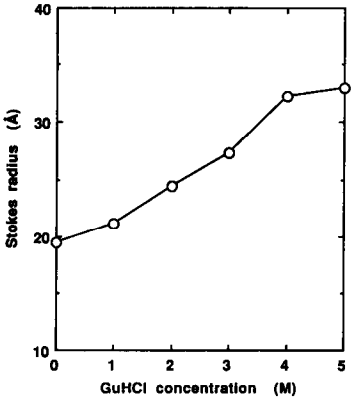


Fig. 5. CD spectra of (solid line) rEGH and (dashed line) pituitary-derived human GH.

Fig. 6. Stokes radius of rEGH at various concentrations of GuHCl. A calibration graph was obtained in the absence of GuHCl by utilizing molecular weight standards of bovine serum albumin (35.5 Å), ovalbumin (30.5 Å), chymotrypsinogen A (20.9 Å) and ribunuclease A (16.4 Å) at a concentration of 0.2 mg/ml.

TABLE IV

MOLECULAR WEIGHT OF EGHs

Methods	Molecular weight (kilodalton)	
	Recombinant EGH	Pituitary-derived EGH
SDS-PAGE (reduced)	25	25
SDS-PAGE (unreduced)	21	21
GFC	23	Monomer[a]
Light scattering	21	ND[b]
Theoretical value	21.327	21.196

[a] From Ref. 16.
[b] ND, not determined.

SDS-PAGE

The apparent masses of rEGH and pituitary-derived EGH given by SDS-PAGE are given in Table IV. The apparent mass of the reduced rEGH was 25 kilodalton, whereas the unreduced rEGH migrated faster (21 kilodalton). This is in agreement with observations on other disulphide-containing proteins [42], and may reflect a more compact SDS–protein complex when disulphide bonds are formed. The co-migration of rEGH with pituitary EGH, under both reduced and unreduced conditions, suggested that rEGH was correctly oxidized with regard to disulphides.

GFC

Recombinant EGH behaved as a monomer (23 kilodalton) in GFC. The result showed good agreement with the previous result for pituitary-derived EGH [16] (Table IV).

As mentioned in the introduction, however, the GFC results could be biased by the chromatographic process. Therefore, to study the dimerization of rEGH and the interaction between rEGH and the GFC support, the apparent Stokes radius of rEGH in GuHCl of various concentrations was determined (Fig. 6). Recombinant EGH was eluted as a single peak in each instance. The Stokes radius of rEGH in the absence of GuHCl was 19.5 Å. The radius increased gradually with increase of GuHCl concentration, with the largest increase at 3–4 *M* GuHCl. At 5 *M* GuHCl, the radius reached 32.9 Å. The shape of this sigmoidal curve for rEGH was similar to that for other proteins [25,43]. The largest increase, at 3–4 *M* GuHCl, corresponds to unfolding of the tertiary structure of rEGH protein, which was detected by fluorescence analysis. Unfolding of the tertiary structure of protein causes a change in the microenvironment of the Trp residue, and this change was detected as a change in the intensity of fluorescence emission and/or a shift of the emission wavelength. A single Trp residue is present in EGH protein (Fig. 2). The emission wavelength was scanned from 300 to 400 nm at an excitation wavelength of 280 nm. The wavelength of maximum emission shifted from 340 to 350 nm between 3 and 4 *M* GuCHl, whereas a significant shift was not observed at other GuHCl concentrations (data not shown).

Dissociation of rEGH dimer and disappearance of the interaction between

rEGH and the GFC support should cause an abnormal decrease and increase in the apparent Stokes radius at certain concentrations of GuHCl, respectively. In Fig. 6, no such decrease or increase is observed. Hence rEGH existed as a monomer and the observed Stokes radius and molecular weight of rEGH in the absence of GuHCl in GFC were not biased by the interaction between rEGH and the GFC support, unless dissociation of rEGH dimer or disappearance of the interaction occurred with unfolding of rEGH protein.

Light scattering

The molecular weight of rEGH was measured by light scattering (Fig. 7). The calculated molecular weight (21 kilodalton) was virtually identical with the theoretical value from the amino acid sequence (Table IV). Oligomerization of protein results in a non-linear line in a Debye plot. A linear relationship, as in this instance, showed the absence of protein oligomerization within the range of protein concentration applied [44,45]. Therefore, the light-scattering results indicated that rEGH exists as a monomer at protein concentrations at least lower than 6.5 mg/ml.

The results of GFC at various concentrations of GuHCl supported the conclusion from GFC without GuHCl that rEGH exists as a monomer. However, we cannot deny the possibility that dissociation of rEGH dimer or disappearance of the interaction between rEGH and the GFC support occured with unfolding of rEGH protein. Also, it is difficult to discuss the effect of rEGH concentration on dimerization because of sample dilution during GFC. Hence it is important to confirm the molecular weight by other methods, such as light scattering or sedimentation equilibrium, although these methods require higher concentrations of protein than in GFC.

Together with the results of the receptor assay by Hirano [33], it is concluded that pituitary-derived EGH also exists as a monomer, because the binding ability of natural EGH would be lower than that of rEGH if oligomerization occurred in natural EGH.

In conclusion, biochemical, biophysical, and immunochemical properties of rEGH were characterized in comparison with pituitary-derived eel, human, bovine,

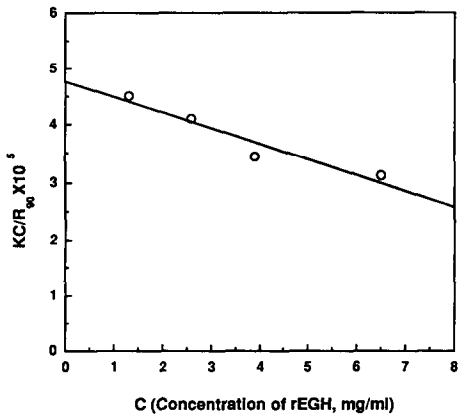


Fig. 7. Debye plots for rEGH. The refractive index of the buffer was 1.3317 and the refractive index increment of the rEGH was 0.1861.

and ovine GHs. The results of N-terminal amino acids sequence analysis, amino acid composition analysis and tryptic mapping revelaed that rEGH had the natural primary structure with an additional Met at the N-terminus. Together with the results of the receptor assay by Hirano [33], the RIA, GFC and light-scattering results suggested that the rEGH was folded into native three-dimensional structure and existed as a monomer, as does pituitary-derived EGH. Other results also supported this conclusion. These data also indicated the usefulness of the large-scale preparation procedure described previously [15].

The large-scale production of *E.coli*-derived rEGH, whose properties are identical with those of the pituitary-derived EGH, will aid future applications of recombinant GH in aquaculture and basic studies on the mode of action of the hormone.

ACKNOWLEDGEMENTS

We are grateful to Professor T. Hirano of the University of Tokyo for performing RIA and for valuable discussions. We thank Dr. S. Nakamura (Otsuka Electronics) for the light-scattering analyses. We also thank Miss Katsuko Shimokawa for skilful assistance.

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