ENHANCEMENT OF THE GROWTH PROMOTING ACTIVITY OF HUMAN GROWTH HORMONE

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SUMMARY: The growth promoting activity of human growth hormone was enhanced at least 4 to 5-fold by controlled digestion with a bacterial proteinase. The increased growth activity was measured in rats by both the tibial line and weight gain assays. By radioimmunoassay the potentiated material was indistinguishable from intact human growth hormone. Pigeon crop sac stimulating activity was increased 4 to 10-fold by the enzymic modification.

Three forms denoted as I, II and III were formed during the digestion. These were separated by chromatography on DEAE-cellulose and all were similar in enhanced biological potencies.

Peptide mapping indicated the three forms lacked residues 138-147 but other structural differences could not be determined by this technique. The activation process involved, therefore, conversion of the hormone from a single chain to a double chain-structure.

Singh et al. (1) isolated from a pituitary extract a modified form of human growth hormone (hGH) that had increased activity when tested by the tibial line assay. This form, denoted as α_3, was present only in small amounts and attempts by us to increase the quantity were unsuccessful. Plasmin has been shown to cleave growth hormones (2) with potentiation of the biological activity of hGH (3) but our experience with preparations of plasmin has been that many do not produce α_3. While looking for an enzyme that would produce better yields of α_3, we found that a "bacterial fibrinolysin" cleaved hGH to produce reproducibly activated hormone. That work is reported here.

MATERIALS AND METHODS

Hormone. The hGH used in the digestions was fraction 3 of step IV of a previously described method (4). Highly purified hGH, free of modified forms, was material obtained in step VII of the same publication and is referred to as intact hGH.

Enzyme. The bacterial fibrinolysin was purchased from Calbiochem, San Diego, California and was used without additional purification.

Digestion. The ratio of enzyme to hGH was 1:100. The digestion was done in 0.1 M sodium phosphate buffer, pH 8.3 for 9 hrs at room temperature. The reaction was stopped with diisopropylfluorophosphate (10^{-3}M) and the solution dialyzed overnight at 5° against 0.025 M sodium phosphate buffer, pH 8.3.

Chromatography on DEAE-Cellulose. (Fig 1). The dialyzed digest was fractionated on DEAE-cellulose (Whatman DE-32) equilibrated with 0.025 M sodium phosphate buffer, pH 8.3 (conductivity 4 mmhos). After application of the sample, the column was washed with the equilibration buffer until all unadsorbed protein was eluted (area A). The buffer was then changed to 0.025 M sodium phosphate buffer, pH 4.3, adjusted to a conductivity of 4 mmho with

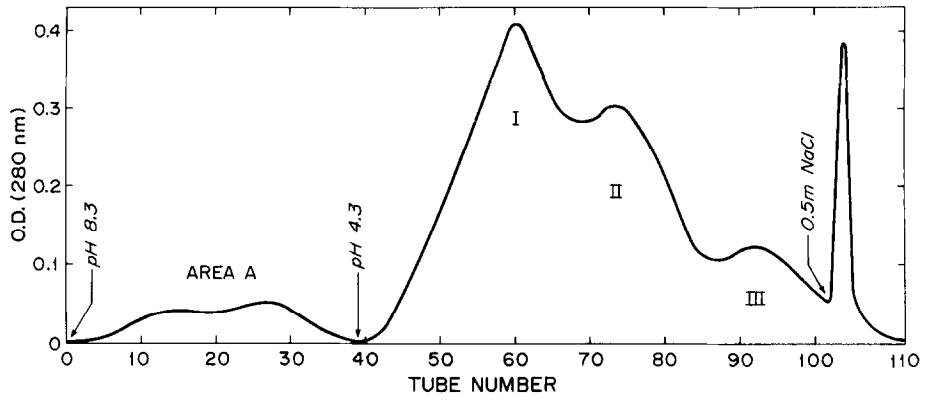

Figure 1. DEAE-cellulose chromatography of 100 mg of hGH after digestion (see text) with the bacterial fibrinolysin. Column size was 0.9 x 15 cm and the flow rate was 1 ml/min. Volume of each tube was 5 ml. Rechromatography of each of the areas, I, II and III, was necessary to obtain homogeneous material.

NaCl. Three peaks, designated as I, II and III were eluted with this buffer. The column was finally washed with the pH 4.3 buffer made 0.5 M in NaCl. To obtain more homogeneous components it was necessary to rechromatograph enriched samples of the three forms.

Electrophoresis. Disc electrophoresis was done at pH 10 (5) and at pH 4 (6). Electrophoresis in sodium dodecyl sulfate (SDS) was performed according to Weber and Osborn (7).

Structure studies. Peptide mapping was by the method of Seavey et al. (8). Amino terminal residues were determined by dansylation (9).

Assays. The tibial line assay was that of Greenspan et al. (10); the weight gain assay was done according to Parlow et al. (11); the pigeon crop sac assay was carried out by the method of Nicoll (12).

Radioimmunoassay for hGH was done as described by Parker et al. (13); human prolactin by the method of Sinha et al. (14).

Protein values for all samples were determined by the procedure of Hartree (15) with bovine serum albumin as the reference.

Studies with plasmin. Four preparations were used: Homolysin (Cutter Laboratories); plasmin-Kabi (Sweden); and two from Lederle Laboratories (Lot #8554B-82 and Lot #4990C-59C). Digestion was carried out as suggested by Ellis et al. (2).

Pituitary extracts. Extracts of human pituitary glands were made with glands collected within 36 hrs after death. The glands were homogenized with 0.05 M sodium bicarbonate buffer, pH 10 (5 ml/g wet tissue); centrifuged at 20,000 X g for 30 min and the supernate examined immediately by disc electrophoresis at pH 10.

RESULTS

Electrophoresis. Fig 2 shows the disc electrophoresis patterns of a fibrinolysin digest of hGH and the purified forms isolated from the digest. Three new components were formed during the proteolysis and all were more

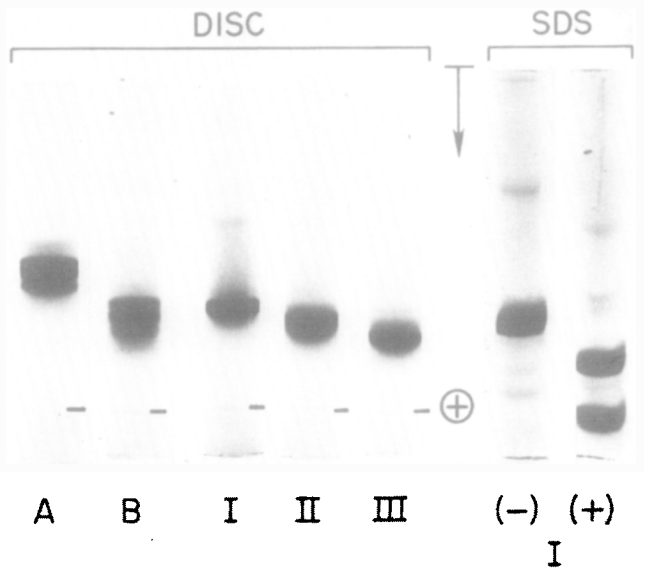

Figure 2. Electrophoresis of Forms I-III. Disc electrophoresis (pH 10, 8% acrylamide): A = hGH used as starting material for the digestion, B = digest made as described in the text. I, II and III are the three forms isolated by DEAE-cellulose chromatography. SDS electrophoresis: I = Form I analyzed without (-) or with (+) mercaptoethanol.

acidic than intact hGH. Prolonged digestion resulted in disappearance of the three components but with formation of no additional bands. Patterns of the purified forms I, II and III show that each was quite homogeneous at pH 10. This was found also at pH 4 (not shown).

When forms I, II and III were examined in SDS in absence of reducing agent they migrated as single bands with molecular weights of 21,000 daltons. After treatment with mercaptoethanol, each dissociated into two fragments of molecular weights of about 15,000 and 7,000 daltons. Since the results were identical for the three forms, only patterns for form I are shown in Fig 2. Intact hGH with or without mercaptoethanol (4) migrated as a single band in SDS.

Bioassays. The modified hGH was first tested in the tibial line assay (Table 1). Forms II and III were given in combination because of insufficient hypophysectomized rats at the time injection was begun. Enzymically altered hGH had a potency near 9 IU/mg whereas intact hGH gave a value that was less than 1 IU/mg.

The modified hGH was next tested in the body weight gain assay. Two assays were performed with modified forms prepared from two different digestions. As seen in Table 2, the altered hGH was at least 4 to 5 times as active as intact hGH.

Table 1. Tibial Line Assay

Sample	Total dose (μg)	Plate width (μ)	Potency* (IU/mg)	95% Limits (IU/mg)	λ
Intact hGH	25	205 ± 4	0.77	0.47 - 1.2	0.22
	100	239 ± 3			
Form I	25	263 ± 4	8.7	4.3 - 48.2	0.3
	100	278 ± 4			
Forms II & III (1:1 mixture)	25	265 ± 6	9.0	4.5 - 47.0	0.29
	100	291 ± 7			
NIH Reference+	25	203 ± 4	(0.92)	(0.73 - 1.28)	---
	100	250 ± 10			

6 rats per dosage group. * Calculated by 2 X 2 design.
+ Bovine GH B-17.

Although the data are not shown, area A from the DEAE-cellulose column (Fig 1) was inactive in the tibial line assay. The material eluted with NaCl showed some activity, probably because residual amounts of the modified forms were in this sample.

Table 3 shows that in the crop sac assay, there was a progressive increase in activity when going from intact hGH, to the partially purified material, to the modified forms.

Radioimmunoassay. No human prolactin was detected in preparations of forms I-III when tested at 1 μg/ml in a radioimmunoassay. A radioimmunoassay for hGH indicated that dilution curves for intact hGH and the modified forms were parallel and not significantly different from each other or from the curve obtained with NIH reference preparation HS-1652 C.

Peptide mapping. Tentative identification of the site of enzymic alteration was obtained by peptide mapping. Maps of the three forms indicated that residues 137-147 had been removed. Mapping did not, however, provide unequivocal data on how the three forms differed. Some maps indicated that digestion removed residues 148 and 149. Sequencing will be required to clarify the differences. Analyses for amino terminal residues were of no help since all three

Table 2. Weight Gain Assay

Sample	Total dose (μg)	10 day wt. gain (g)	Potency* (IU/mg)	95% Limits (IU/mg)	λ
Intact hGH	20	20.0 ± 2.5	1.1	0.6 - 2.1	0.29
	100	30.2 ± 2.1			
Form I	5	20.3 ± 1.8	4.6	3.0 - 7.5	0.22
	25	30.4 ± 1.1			
Forms I & II (1:1 mixture)	5	26.8 ± 2.7	10.1	4.6 - 40	0.39
	25	31.5 ± 2.1			
Reference+	20	17.4 ± 0.6	(0.92)	(0.73 - 1.28)	----
	100	30.0 ± 2			
Intact hGH	25	19.4 ± 1.3	0.91	0.67 - 1.2	0.15
	100	34.7 ± 1.8			
Form I	5	20.4 ± 1.2	3.9	2.9 - 5.3	0.15
	20	31.0 ± 0.13			
Form II	5	20.7 ± 0.7	5.1	3.7 - 7.2	0.17
	20	35.8 ± 2.6			
Form III	5	21.1 ± 1.0	3.7	2.5 - 5.4	0.19
	20	29.8 ± 2.0			
Reference+	25	18.0 ± 1.8	(0.92)	(0.73 - 1.28)	----
	100	36.3 ± 2.0			

6 rats per dosage group. * Calculated by 2 X 2 design.

+ NIH bovine GH (B-17).

forms gave only phenylalanine as an amino terminus.

Digestion with plasmin. Of the four preparations of plasmin tested, only Lot #8554B-82 from Lederle Laboratories produced material that behaved electrophoretically as α_3-hGH.

Pituitary extracts. Disc electrophoresis of the extracts at pH 10 detected only intact hGH and a small amount of a component that migrated as a desamido

Table 3. Crop Sac Assays

Sample	Total dose (μg)	Mucosal weight (mg)	Potency* (IU/mg)	95% Limits (IU/mg)	λ
Intact hGH	16	13.3 ± 0.9	1.6	1.1 - 2.3	0.17
	80	24.1 ± 1.6			
Reference+	2	16.2 ± 1.5	(26)	(22 - 31)	----
	10	32.9 ± 1.4			
Partially purified hGH	8	16.9 ± 1.3	2.5	1.1 - 4.3	0.27
	40	25.0 ± 2.6			
Reference+	2	20.6 ± 1.2	(26)	(22-31)	----
	10	36.0 ± 1.9			
Form I	8	19.9 ± 0.9	17.1	7.9 - 62	0.39
	40	28.9 ± 2.8			
Reference+	2	15.4 ± 1.2	(26)	(22 - 31)	----
	10	23.6 ± 2.2			
Form II	4	15.8 ± 0.6	12.9	6.4 - 25	0.3
	20	31.4 ± 3.9			
Reference+	2	17.9 ± 0.6	(26)	(22 - 31)	----
	10	30.0 ± 3.4			
Form III	8	20.4 ± 1.9	6.7	3.0 - 15.1	0.37
	40	31.6 ± 3.5			
Reference+	2	17.9 ± 2.3	(26)	(22 - 31)	----
	10	33.6 ± 3.5			

6 rats per dosage group. * Calculated by 2 X 2 design.

+ Ovine prolactin S-10 (26 IU/mg).

form. Faster migrating components were absent. After the extract stood for one week at 5°, components in the α_3 area appeared.

DISCUSSION

The alteration produced by the bacterial proteinase is similar but not identical to that noted for α_3 isolated from pituitary extracts (1). The α_3, which also had enhanced biological activity, lacked residues 135-146 whereas

the forms produced by the bacterial enzyme were missing residues 137-147 and possibly residues 148 and 149. For potentiation of growth promoting activity, therefore, the cleavage must remove at least 11 residues. The form lacking only residues 135-140 (1,16,17) does not show enhanced growth promoting activity. Pigeon crop sac stimulating activity, on the other hand, is potentiated when even a single cleavage is made in the hGH chain (4).

Since three of the four plasmin preparations did not produce α_3-like forms, this enzyme is probably not the one responsible for the activation of hGH in pituitary extracts and suggests that some plasmin preparations are contaminated with other proteinases.

That the biological activity of hGH can be potentiated by enzymic alteration supports the suggestion (18) that the hormone may have to be modified post-synthetically to exert its physiological effects. The hypothesis herein proposed is that the ribosome forms hGH as an intact 191 amino acid sequence, the storage form. During secretion, deletion of sequences occurs resulting in activation. Failure of the intact form to be so activated argues that this is a pituitary secretory function, and our inability to demonstrate cleaved forms in fresh glands supports this hypothesis.

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