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Efficacy Tests of Recombinant Human Growth Hormone Produced from *Saccharomyces cerevisiae*

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Abstract: The potency of yeast-derived methionyl-free human growth hormone (rhGH), which was obtained by removal of the N-terminal Met from methionyl-hGH, was estimated by *in vitro* and *in vivo* assays. In radio-receptor assay where the binding affinity of growth hormone to the receptor was estimated, the recombinant hGH showed 2.9 international units (IU) per mg of specific activity. In contrast, pituitary-derived human growth hormone had a slightly lower receptor binding activity (2.5 IU/mg) compared with recombinant growth hormone. For the *in vivo* assay, efficacy of rhGH was tested by use of hypophysectomized rats, in which pituitary organs were surgically removed, resulting in the termination of growth hormone secretion. The weight-increase in rats by the injection of rhGH was almost identical to the result obtained by the injection of the same amount of pituitary-derived (international standard) hGH. A comparision of the secondary structures of rhGH and rMet-hGH by circular dichroism spectrophotometer demonstrated that the removal of the methionyl residue from rMet-hGH did not exert any effect on the structure of the growth hormone. In conclusion, methionyl-free human growth hormone produced from yeast was highly potent in biological activity and maintained a legitimate three dimensional structure.

Key words: CD structure, human growth hormone, hypophysectomized rat, radio receptor assay, weight-gain assay.

Human growth hormone (hGH) is produced in the pituitary gland and plays a pivotal role in the growth of cartilage bone during the pre-adolescent period of human beings. The deficiency of growth hormone inevitably leads to short stature. The first successful clinical trial of hGH was demonstrated by Raben to treat pituitary dwarfism patients in 1958. Since then the demand for natural hGH substantially increased until cases of Creutzfeldt-Jacob disease were reported around the world. This rare, but critical neurological disease was caused by contaminating material in the pituitary-derived hGH obtained from human cadavers (Aldhous, 1992). Since the end of the 1980s, the biosynthetic hGH produced by recombinant microorganisms has replaced the natural growth hormone.

Natural human growth hormone is composed of 191 amino acids. When expressed in the recombinant *E. coli*, the N-terminal methionyl residue, translated from the transcriptional start codon, is not removed, thereby resulting in the methionyl-human growth hormone (rMet-hGH) with 192 amino acids (Olson *et al.*, 1981). It was reported that the growth promoting activity of *E. coli*-derived Met-hGH was equally as effective as that of natural human growth hormone (Flodh, 1987). However, many clinical reports demonstrated that the methionyl residue at the N-terminus of rMet-hGH was suspected of to initiate antibody formation in many of the patients treated (Takano *et al.*, 1986; Tyllstrom *et al.*, 1985). Therefore, biotech companies around world worked to remove the additional methionine from rMet-hGH. Since then, recombinant methionyl-free hGH from *E. coli* became available for therapeutic treatment.

As reported earlier, recombinant Met-hGH has been expressed in *Saccharomyces cerevisiae* (Cho *et al.*, 1988) and its physico-chemical properties thoroughly tested. After fermentation the majority of the rMet-hGH molecules were obtained in the form of insoluble aggregates in yeast cells and thus an elaborate refolding procedure was necessary to restore the full biological potency (Won *et al.*, 1991). Extensive clinical trials proved that rMet-hGH from yeast was fully potent in growth promoting activity when used to treat pituitary dwarfism patients (Kim, 1992; Lee *et al.*, 1992; Seo, 1991).

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Ltd. has successfully developed recombinant methionyl-free hGH (rhGH) from *Saccharomyces cerevisiae*. Worldwide this was the first recombinant methionyl-free hGH from yeast ever developed for a therapeutic purpose (Park *et al.*, 1994). In order to remove selectively the N-terminal Met from rMet-hGH during purification, a novel aminopeptidase was isolated and obtained in large quantity from soil bacterium. The purpose of this research is to determine the biological efficacy of rhGH from yeast. Furthermore, a structural study by means of spectroscopy is also described.

Materials and Methods

Human growth hormone

The purified rhGH and rMet-hGH from yeast (*Saccharomyces cerevisiae*) were obtained from LG Chem. Pharmaceutical Division. The purification methods and biological efficacy of rMet-hGH from yeast were reported previously (Won *et al.*, 1991; Park *et al.*, 1990). Recombinant methionyl-free hGH was produced from rMet-hGH by treatment with aminopeptidase in the midtst of purification (Park *et al.*, 1994). Therefore, rMet-hGH and rhGH originated from yeast cells harboring the same vector. A commercially available rhGH expressed in *E. coli* was purchased from a local pharmacy.

International standard hGH (IS-hGH) purified from human pituitary gland was obtained from NIBSC (National Institute for Biological Standards and Control, WHO). The specific activity of IS-hGH was 2.506 IU/mg according to radio-receptor assay. In receptor binding assay, hGH standard (NIDDK-NIH AFP-4793B) was used for iodination.

Hypophysectomy

The hypophysectomized rats were obtained by removal of the pituitary gland from Sprague Dawley rats. The method used for the removal of the pituitary gland was the parapharyngeal method which has been described in detail elsewhere (Waynforth, 1980).

Briefly, a hole was drilled in the base of the cranium of an experimental rat of body weight 150~200 g after a midline incision was made along the length of the neck up to the point of the lower jar. Once exposed, the pituitary was sucked out with a pasteur pipet, curved at the end, which was connected to a vacuum source. To ensure a low mortality rate from hypophysectomy carried out by the operation, postoperative care was followed as advised.

Weight-gain assay

The biological potency of growth hormone was assessed by measuring the increase in weight of hypophysectomized rats. Groups of ten rats each were used for the daily subcutaneous injection of either 0.9% saline, 30 μg of IS-hGH, 30 μg of rhGH, or 90 μg of rhGH. The weight of the rats was measured every day, and the mean cumulative weight gain vs treatment day was plotted for each group.

Radio-receptor assay

The *in vitro* biological activity of the growth hormone was determined by receptor binding assay. The growth hormone receptor fractions were obtained from pregnant rabbit liver membranes as decribed by Tsushima *et al.* (1976). Iodination of hGH was based on the method by Thorell and Johnson (1971) with some modifications. The experimental procedure and the method to calculate the specific activity of the growth hormone have been described in detail elsewhere (Park *et al.*, 1990).

Circular dichroism spectroscopy

Circular dichroism spectra of the growth hormone were measured by a JASCO-600 CD spectrophotometer. The CD spectrum was calibrated by use of d-10-camphorsulfonic acid. The protein was dissolved in 25 mM potassium-phosphate buffer at pH 7.5. The average molecular weight of 115 was used for the mean ellipticity calculation. The protein spectrum was obtained from the average of three scannings from which the buffer spectrum was subtracted.

HPLC

Reverse-phase HPLC was used to evaluate the purity of the experimental materials. A C_{18} U-Bondapak (4.5 ×300 mm) RP-column was equilibrated with 20% (v/v) acetonitrile/0.05% trifluoroacetic acid in deionized water. The lyophilized growth hormone was dissolved in 270 mM glycine-0.6 mM sodium phosphate at pH 7.4 and then injected to the column. The concentration of acetonitrile was increased linearly up to 80% over 40 min. The elution profile was monitored at 280 nm.

Results

Puritiy assessment

Prior to *in vitro* and *in vivo* experiments, the purity of rhGH obtained was determined by C_{18} reverse-phase HPLC. Fig. 1 shows the HPLC result of rhGH. As acetonitrile concentration was incresed, a single symmetrical protein peak was observed. It was concluded from the experiment that the protein was highly homogeneous and pure. We obtained virtually the same chromatographic pattern with the same retention time by use of rMet-hGH (data not shown). At the experimental

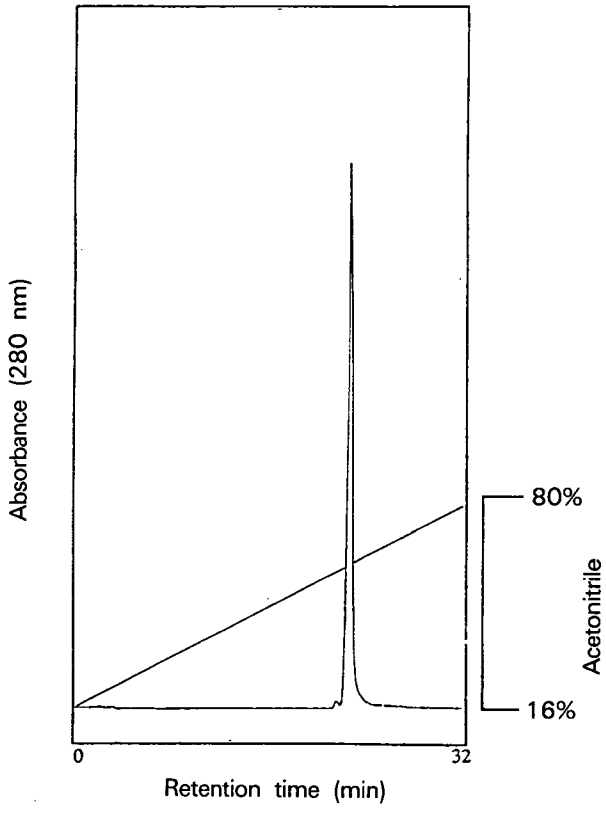

Fig. 1. C_{18} reverse-phase HPLC profile of rHGH. The protein solution was applied to C_{18} μ-Bondapack HPLC column equilibrated with 20% (v/v) acetonitrile/0.05% TFA in deionized water. The organic solvent was linearly increased with a flow rate of 1.0 ml/min.

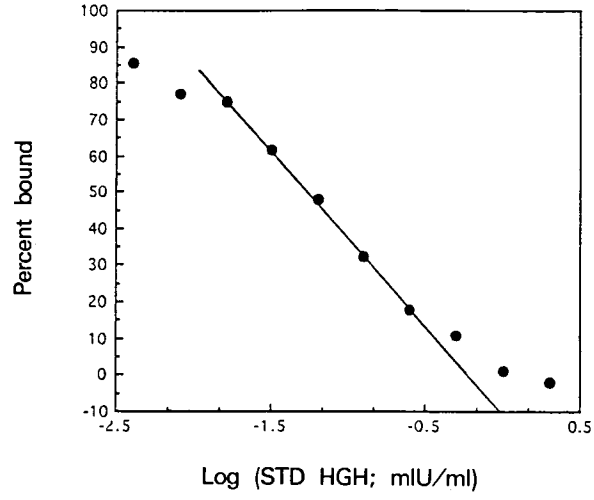

Fig. 2. Regression curve of radio-receptor assay. The X-axis represents the concentration of hGH (mIU/ml) in logarithmic scale and the Y-axis indicates the percentage of ^{125}I-hGH bound to the receptors. The points where linearity hold are used for the calculation of receptor binding activity of hGHs tested.

Table 1. Activity determination of growth hormones by radio-receptor assay

Materials[a]	IS-hGH	rMet-hGH	rhGH
Specific activity[b]	2.506	2.904	2.895

[a] IS-hGH, rMet-hGH, and rhGH indicate respectively pituitary-derived hGH, yeast-derived methionyl-hGH, and yeast-derived hGH.
[b] For the calculation of specific activity, protein concentration was determined by TCA-Lowry method (Bensadon and Weinstein, 1976) using Bovine serum albumin (Biorad, USA) as a standard. The specific activity was obtained by average of five different experiments.

conditions used, rMet-hGH and rhGH were not separated. It was reported that rMet-hGH and rhGH could be separated by use of a silica-based C18 resin at neutral pH and elevated temperatures (e.g. 45℃) (Welinder *et al.*, 1987). Because the aim of the HPLC experiment was only to identify the homogeneity of the test material, the experiment shown in Fig. 1 was enough to serve our purpose and therefore we did not further try to resolve rMet-hGH and rhGH by HPLC.

Radio-receptor assay

When the concentration of hGH was increased in the test tubes, ^{125}I-hGHs bound to hGH receptors in the liver membrane fractions were displaced, resulting in the sigmoidal standard curve shown as Fig. 2. From the linear portion of the standard curve, one can calculate the activity of the growth hormone to be tested (Park *et al.*, 1990 for detailed calculation method). This competition assay is used routinely for the determination of *in vitro* specific activity of growth hormones.

Table 1 displays the specific activites of the various growth hormones tested. The specific activity of rMet-hGH was the same as that of rhGH within standard deviation. Therefore, it can be concluded that the N-terminal methionine attached to rhGH does not have any effect on the binding interaction between the growth hormone and its receptors. It is also concluded that the method to produce rhGH from rMet-hGH did not affect any receptor binding activity of the protein. However, the specific activity of pituitary-derived hGH (IS-hGH in Table 1) was somewhat lower than the biosynthetic growth hormones.

Weight gain assay

Hypophysectomized rats do not produce growth hormones and thus they are good animal models for pituitary dwarfism. As shown in Fig. 3, the rats injected with saline as a control did not grow, whereas the weight of the rats injected with rhGH increased linearly. The slight increase of weight shown in the control group was due to the residual plasma growth hormones. The weight increase in hypophysectomized rats resulted from longitudinal bone growth. Tibia measurement after

Table 2. Tibia test in hypophysectomized rats

Group	Saline	IS-hGH (30 μg)	rhGH (30 μg)	rhGH (90 μg)
Mean± S.E. (mm)	30.41 ± 0.42	32.48 ± 0.34	32.59 ± 0.33	33.45 ± 0.14

IS-hGH and rhGH refer to international standard growth hormone and recombinant methionyl-free growth hormone, respectively.

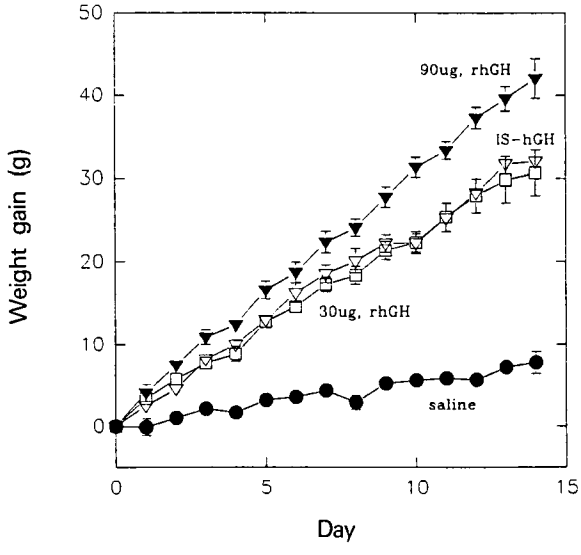

Fig. 3. Weight gain observed in hypophysectomized rats. Data represented as open circles was obtained from the daily injections of 0.9% saline. Open triangles and closed triangles were obtained from the injections 30 mg of rhGH and IS-hGH, respectively. Closed circles was from 90 mg injections of rhGH. Results were expressed as the mean of 10 rats in each group.

the 14th day of the experiment demonstrated a strong correlation between weight increase and tibia growth (Table 2).

We also observed dose-response growth promoting activity by rhGH. A higher dosage of rhGH promoted a substantially higher weight increase. As indicated in Fig. 3, the growth promoting activity of rhGH could not be distinguished from that of pituitary-derived hGH within experimental errors when the same amount of hGH was injected. Though the hypophysectomized rat is known to be immunologically deficient (Istan *et al.*, 1981), in Fig. 3 we saw no evidence of early plateauing of response that would indicate an exaggerated immunological response to growth hormone.

Hypoglycemic effect

Besides growth promoting activity, growth hormones exert many anabolic events. It is known that hGH has insulin-like effects. In GH-deficient subjects, growth hormone produces transient insulin-like effects, including the induction of hypoglycemia, resulting from the stimulation of glucose uptake and utilization by peripheral tissues and the inhibition of lipolysis. This can be tested in growth hormone deficient animals by measuring blood glucose after GH administration.

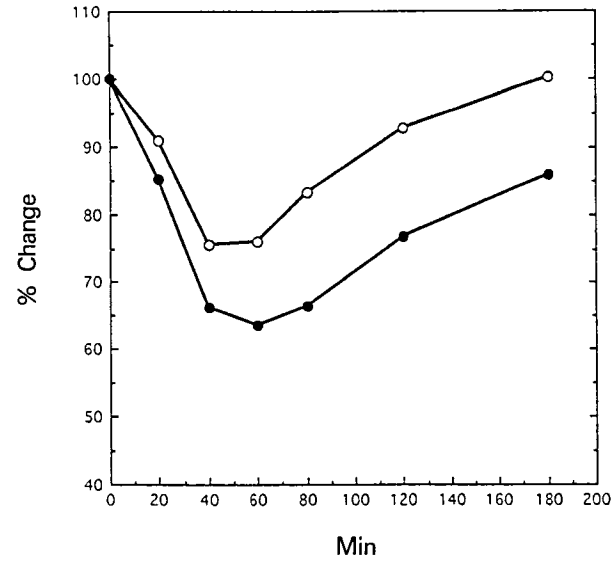

Fig. 4. Acute hypoglycemic effect of human growth hormone. The hypophysectomized rats were administered with 30 μg (open circles) and 90 μg (closed circles) of rhGH, respectively. Blood glucose was measured from the serum at regular intervals. Injection of saline to the rats did not induce any change of blood glucose within the experimental fluctuations.

As shown in Fig. 4, a decrease in the blood sugar was observed after 30~40 min of rhGH injection in hypophysectomized rats. After another 30~40 min, blood glucose was back to normal. Fig. 4 also shows that the hypoglycemic effect of hGH is dose-related. The dose-response hypoglycemic phenomenon was well correlated with that observed in the weight-gain experiment.

CD spectrum

In order to determine the three dimensional structure of yeast-derived rhGH and also to test whether or not the structure of rhGH was affected during the N-terminal methione removal process, CD spectra were measured. Secondary structure calculation from CD spectrum showed that rhGH from yeast consisted of about 55% α-helix. As demonstrated in Fig. 5, the CD spectrum of rhGH was virtually the same as that of rMet-hGH, indicating that N-terminal methionine did not have any effect on the authentic secondary structure of rhGH. It was also concluded from the figure that the production conditions of rhGH did not affect the legitimate tertiary structure of the growth hormone.

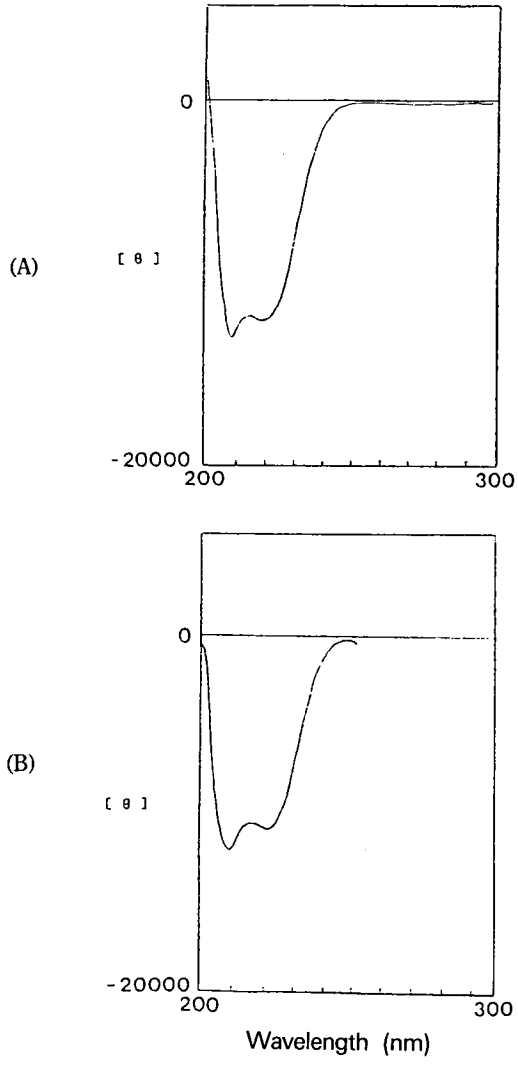

Fig. 5. Circular dichroism spectra of rhGHs. The experimental conditions were described in Materials and Methods. Plot A and plot B represent, respectively, CD spectra of rhGH and rMet-hGH.

Discussion

Growth hormone is secreted by acidophilic cells of the anterior pituitary. The major growth-promoting role of growth hormone is its ability to stimulate the release of insulin-like growth factors from the liver (Goodman, 1968). Growth hormone has a number of metabolic functions apparently unrelated to its role in growth. The most important of these is its stimulation of lipolysis in adipose tissue during prolonged starvation, exercise, and chronic hypoglycemia (Beck *et al.*, 1957).

As a host system for producing recombinant growth hormones, microorganisms such as *E. coli* or *Saccharomyces cerevisiae* can be used efficiently. Although the expression level of growth hormones during fermentation is quite similar in both systems, they are quite different in terms of post-translational modifications. In *E. coli*-derived rMet-hGH, a sulfoxide was observed at Met14 in addition to two deaminations at Asn149 and Asn152 (Becker *et al.*, 1988). Another experiment showed that a Cys182-Cys189 trisulphide bridge could be observed in *E. coli*-originated rhGH (Jespersen *et al.*, 1994). However, we found that in rMet-hGH from yeast cells minor portions of N-terminal Met was modified to methionyl sulfoxide (manuscript in preparation). Therefore, it is necessary to test extensively the physicochemical properties and biological activities of the purified rhGH produced from yeast cells.

Several bioassay procedures are commonly used to estimate the potency of growth hormone. The procedures include changes in the length of the tibia, in epiphyseal width, and in weight gain. Of the three methods, weight-gain assay by use of hypophysectomized rats is the most widely used *in vivo* test. For *in vitro* assay, receptor binding assay and radioimmuno assay can be used. However, immuno assay is not as sensitive as the radio-receptor assay when one wants to evaluate the effect of structural variation of growth hormone on the receptor binding affinity. In general, weight-gain assay as an *in vivo* test and radio-receptor assay as an *in vitro* test are routinely used for quality control assessment of recombinant growth hormones.

As demonstrated in the receptor binding assay, the specific activity of yeast-derived rhGH was 2.9 IU/mg of the protein. This value was among the highest obtained with recombinant growth hormones so far, suggesting that the legitimate tertiary structure was obtained during the refolding process. Besides the legitimate structure, it is judged from Fig. 1 that the purity of the experimental material may contribute to the high specific activity of yeast-derived hGH. In Table 1, the specific activity of rMet-hGH was the same as that of rhGH. Similar results were reported from the experiments with *E. coli*-derived human growth hormones.

It is also evident from table 1 that both rMet-hGH and rhGH are more potent than the pituitary-derived hGH in the binding of the growth hormone receptor. It is known that pituitary-derived hGH is somewhat heterogeneous (Goodman *et al.*, 1972). In addtion to the major 22 kDa hGH molecules with 191 amino acids, it contains both 20 kDa hGH in which a portion of the peptide is deleted (Kostyo *et al.*, 1987), and hGH molecules with modified amino acids at certain positions (Becker *et al.*, 1988). These heterogenous fractions of natural growth hormones may have lower specific activities compared to the homogenous authentic growth hormones. Although 20 kDa hGH variant has growth-promoting activity similar to that of 22 kDa hGH, there has been a report that 20 kDa hGH had only 10~30 % of the ability of hGH to compete for binding sites on fat cells, or to stimulate glucose oxida-

tion and lipolysis (Goodman *et al.*, 1986).

Fig. 2 demonstrates that pituitary-derived hGH and yeast-derived hGH produce almost identical weight increase in hypophysectomized rats. Tibia length observed in Table 2 indicates that the weight gain by rhGH was due to longitudinal bone growth. The results from Table 1 and Fig. 2 further indicate that aminopeptidase treatment of rMet-hGH to obtain rhGH did not modify the structure of the growth hormone molecule. This conclusion was supported by the secondary structure determination (Fig. 3). Fig. 3 demonstrates that the CD spectrum of rMet-hGH was essentially identical to that of rhGH. As judged from two minima at 208 nm and 220 nm of the rhGH CD spectrum, the protein is composed of an α-helix. The content of the α-helix agrees well with the value calculated from X-ray crystal structure of *E. coli*-derived rhGH (De Vos *et al.*, 1992) indicating that refolding of rhGH during purification fully restored authentic three dimensional structure. Finally, the experiemts described in this manuscript demonstrate that yeast-derived methionyl-free human growth hormone is very potent in receptor binding and also *in vivo* growth promoting activity.

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