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Single-Step Purification of Recombinant Human Growth Hormone (hGH) Directly from Bacterial Osmotic Shock Fluids, for the Purpose of ^{125}I-hGH Preparation[1]

Maria Teresa C. P. Ribela, Iara M. C. Camargo, João E. Oliveira, and Paolo Bartolini
Department of Application of Nuclear Techniques in Biological Sciences, IPEN-CNEN, 05508 São Paulo, Brazil

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A good quality tracer, to be used in the radioimmunoassay of human growth hormone, was prepared by applying the chloramine-T iodination technique to the recombinant product obtained after a single-step high-performance size-exclusion chromatography purification of a bacterial osmotic shock fluid. The labeling reaction presented a yield of about 65% and the purified tracer exhibited an antibody binding of ~50% (NIDDK reference antiserum diluted 1:600,000). These values are very similar to those obtained by radioiodinating highly purified clinical-grade recombinant human growth hormone obtained from the same periplasmic extract after the regular six-step purification process. Both tracers provided the same accuracy, when evaluated with the use of commercial-quality control samples in a classical radioimmunoassay methodology, their stability being practically identical: about 18% decrease in antibody binding after 2 months of storage at −20°C. The novel approach permits the utilization of transformed *Escherichia coli* strains as a source of freshly prepared, radioiodination-grade recombinant proteins, capable of providing better reproducibility and reagent continuity.

Key Words: **human growth hormone (hGH); DNA recombinant; *E. coli;* osmotic shock fluid; high-performance size-exclusion chromatography (HPSEC); radioiodination.**

Radioimmunoassay (RIA),[2] immunoradiometric assay (IRMA), and radioreceptor assay (RRA) are still widely used tools for protein hormone determination and binding studies in a variety of applications. These include insulin (1), calcitonin (2), prolactin (3–6), thyrotropin (7,8), luteinizing hormone (5), and follicle-stimulating hormone (5,9). Regarding the last hormone, in a recent International Collaborative Study organized by the WHO for the definition of international standards of urinary and recombinant hFSH, 68% of the participating laboratories used either RIA or IRMA systems (10). Concerning specifically growth hormone (GH), the object of the present work, its radioiodination for RIA (11–14), RRA (15), or tracer-spiking experiments (16) greatly exceeds its utilization in nonradioisotopic systems.

The success of all these assays depends on reagent quality, which determine specificity and sensitivity, the labeled protein being one of the most critical reagents (17). The availability, quality, continuity, and cost of an iodination-grade hormone or antibody preparation is therefore one of the most important aspects to be considered. Recombinant DNA technology has provided practically unlimited amounts of genetically engineered proteins that can be extremely useful in this respect. In previous studies, the suitability of the use of DNA recombinant products for radioiodination and standard preparation was reported (18,19). Authentic recombinant human growth hormone (rec-hGH) and thyrotropin, which were purified by several

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[2] Abbreviations used: RIA, radioimmunoassay; IRMA, immunoradiometric assay; RRA, radioreceptor assay; rec-hGH, recombinant human growth hormone; hFSH, human follicle-stimulating hormone; HPSEC, high-performance size-exclusion chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; BSA, bovine serum albumin.

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chromatographic steps, provided stable, high specific activity tracers, identical to the best pituitary preparations. In previous work, rec-hGH was also analyzed directly in osmotic shock fluids to evaluate expression yield and product quality prior to its purification (20). For this purpose reversed-phase high-performance liquid chromatography (RP-HPLC) and high-performance size-exclusion liquid chromatography (HPSEC) were utilized.

In the present study a one-step HPSEC separation technique was applied immediately after obtaining the osmotic shock fluid, in order to prepare iodination-grade rec-hGH. The radioiodinated tracer thus obtained was compared, in terms of quality and applicability, to the analogous product obtained from the same periplasmic extract after the regular six-step purification process. The same methodology, with the necessary modifications, could also be applied to the preparation of conventional or genetically engineered radioiodinated monoclonal antibodies.

MATERIAL AND METHODS

Starting Material

The periplasmic–osmotic shock fluid used as the starting material for hGH purification was prepared from *Escherichia coli* K12, RRI strain harboring an expression vector, constructed in our laboratory, in which the hGH gene (cDNA) was under control of the λ P_L promoter. Transformed *E. coli* was grown at 30°C, under selective conditions (12.5 μg/mL of tetracycline), in a 20-L laboratory bioreactor (New MBR, Zurich, Switzerland), using a complex culture medium, which was a twofold concentrate of the HKSII medium described by Jensen and Carlsen (21) and a glucose feeding rate of 1.2 g L^{-1} h^{-1}. After about 5 h, having reached an absorbance of about 5 A_{600} units, activation was carried out at 42°C for 6 h.

E. coli osmotic shock was carried out according to the method of Koshland and Botstein (22). Briefly, 5 L fermentation broth with 8.0 A_{600} units was centrifuged at 4°C and 6000*g* for 10 min. The pellets were resuspended in 400 mL of ice-cold 10 mM Tris–HCl, pH 7.5, containing 20% (w/v) sucrose. Then 13.3 mL of 0.5 M EDTA, pH 8.0, was added and incubation on ice was carried out for 10 min. The cells were then centrifuged again and the pellet was resuspended by vigorous agitation in 200 mL of cold 1 mM Tris–HCl, pH 7.5, solution. The mixture was incubated for 10 min on ice and then centrifuged again for 15 min. The supernatant was removed and saved as the periplasmic fraction.

Chemicals and Reagents

All chemicals were analytical reagent grade, purchased from Merck (São Paulo, Brazil) and Sigma (St. Louis, MO).

The First International Standard for Somatropin (rec-hGH) coded 88/624, utilized in HPLC studies, was kindly donated by the National Institute for Biological Standards and Control (South Mimms, UK). Pituitary human growth hormone reference preparation (NIDDK-hGH-RP-1), anti-hGH antiserum (NIDDK-anti-hGH-2), and highly purified pituitary human growth hormone (pit-hGH) for iodination (NIDDK-hGH-I-3) were kindly provided by the National Hormone and Pituitary Program (Baltimore, MD). Rec-hGH, batch 06297, secreted in *E. coli* periplasmic space, was extracted by osmotic shock procedure and purified in this laboratory (20). Anti-rabbit IgG second antibody produced in sheep was also from this laboratory (IPEN, São Paulo, Brazil).

Na ^{125}I free of carriers and reductant was purchased from MDS Nordion (Kanata, Ontario, Canada) and Sephadex G-100 was from Pharmacia (São Paulo, Brazil). Human blood-based immunoassay quality controls (Dade Tri-level) were purchased from Baxter Diagnostic (Deerfield, IL).

Single-Step Rec-hGH Purification, by HPSEC

A Shimadzu Model SCL-10A HPLC apparatus with a SPD-10AV UV detector was utilized for processing 200-μL aliquots of periplasmic fraction on a Tosohaas (Montgomeryville, PA) G2000SW column (60 cm $\times$ 7.5 mm i.d., particle size of 10 μm, and pore size of 125 Å) coupled to a 7.5 cm $\times$ 7.5-mm i.d. SW guard column. Fractions of 150 μL were collected across the peak corresponding to rec-hGH. The mobile phase was 0.025 M ammonium bicarbonate, pH 7.0, with a flow rate of 1.0 mL/min. This purification process normally provides ~95% yield of rec-hGH, with a specific activity (purity) of ~45%.

Regular Rec-hGH Six-Step Purification Process

The product was purified in six sequential steps that provided clinical-grade rec-hGH: one precipitation, two gel-filtration columns, two ion-exchange columns, and one hydrophobic interaction chromatography step were used. This purification process normally provides ~40% yield of rec-hGH, of 100% purity (23).

Radioiodination

The ^{125}I-labeling of rec-hGH was carried out employing a modification of the original chloramine-T technique, described in previous work (18), which employs only 0.8 μg of chloramine-T and 1.0 μg of sodium met-

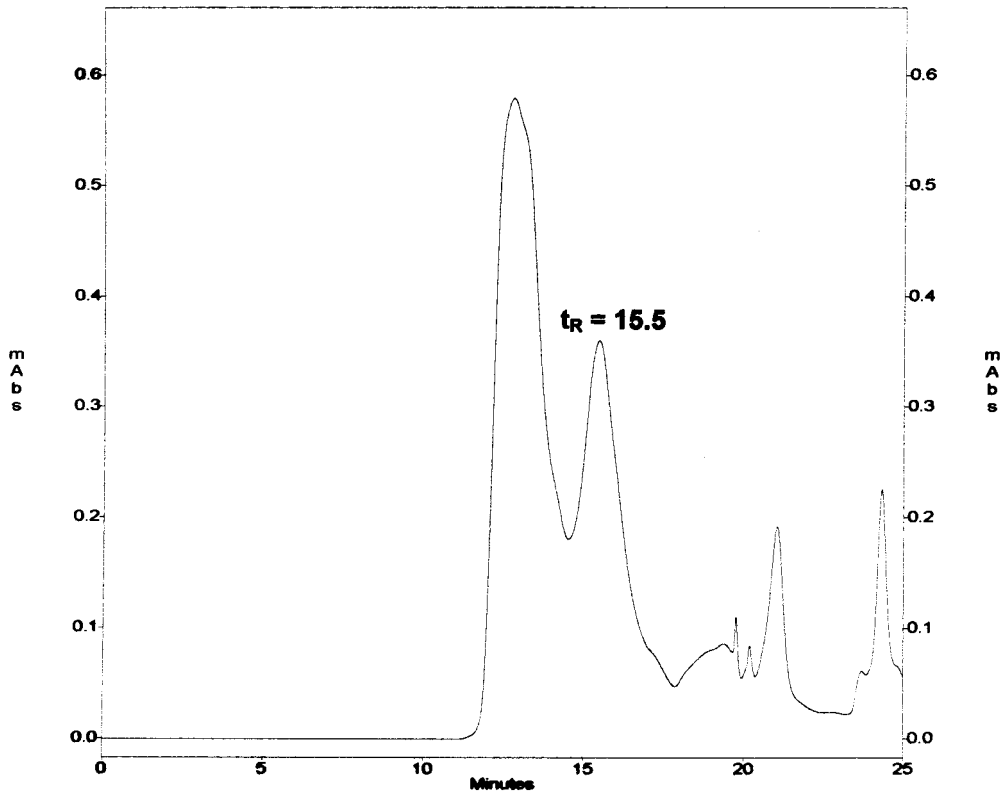

FIG. 1. HPSEC on a G2000 SW column of an osmotic shock fluid obtained from an hGH-secreting *E. coli* strain. A parallel run of the First International Standard of rec-hGH (88/624) indicated a t_R of 15.2 min; mAbs, milliabsorbance units with detector wavelength at 220 nm.

abisulfite. When rec-hGH was obtained as described in the single-step purification, 12–15 MBq (324–405 μCi) and about 2–4 μg of hormone (50 μl of either the central fraction or the whole pooled peak) was employed. When rec-hGH was obtained according to the regular six-step purification process, 32 MBq (865 μCi) of radioisotope and 10 μg of hormone (10 μl) were employed. Iodinated hormones were purified by gel filtration on a Sephadex G-100 column (18).

Radioimmunoassay

RIA procedures were performed by incubating anti-hGH antibody (100 μL/tube, final dilution 1:600,000) in 0.01 M sodium phosphate, pH 7.6, 0.1% BSA, 0.1% Tween 20 (RIA buffer) with 25,000 cpm tracer diluted in 100 μL RIA buffer, in the presence or absence of unlabelled hormone as competitor (100 μL). After overnight incubation at 4°C, bound and unbound hormone were separated by incubation for 2 h, at room temperature, with 100 μL of second antibody (diluted 1:10), followed by centrifugation at 3000*g* for 20 min. In each assay low-, intermediate-, and high-level quality control preparations were used.

Antiserum titration curves were carried out using the same methodology, with antibody dilutions ranging from 1:10^5 to 1:2 $\times$ 10^6.

RP-HPLC

RP-HPLC was employed to determine rec-hGH in the periplasmic fraction as well as in the HPSEC eluted fractions, as previously described (20). Quantification was carried out against the international standard of rec-hGH.

Protein Determination

Total protein content of the extracts and purified fractions was determined by the classical method of Lowry *et al.* (24).

RESULTS

Figure 1 shows the HPSEC chromatogram of an osmotic shock fluid obtained from the bacterial strain transformed with an hGH expression vector. This bacterial culture contained 1.17 mg rec-hGH/g wet wt cells (12.8 mg rec-hGH/L), while in the osmotic shock fluid the protein of interest was present at a concentration of 200 μg/mL, representing 7.5% of the total protein con-

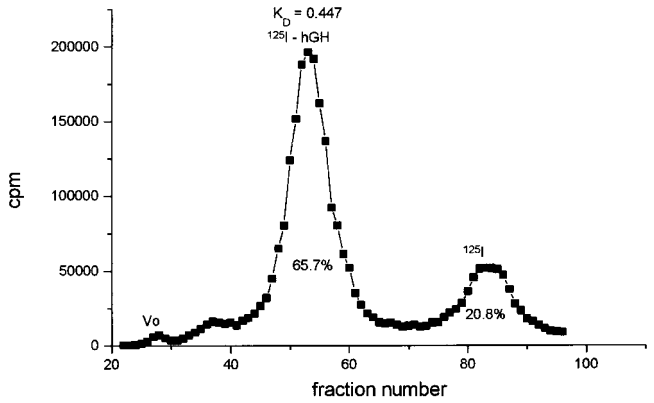

FIG. 2. Purification and analysis of tracer A on a 50 × 2.5-cm i.d. Sephadex G-100 column; flow rate 12 mL/h.

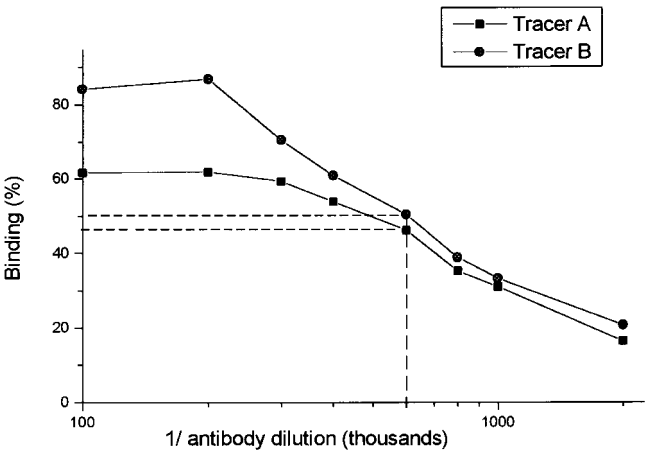

FIG. 3. Antiserum titration curves for ^{125}I-hGH directly prepared from an osmotic shock fluid (tracer A) and ^{125}I-hGH obtained by radioiodination of a highly purified product (tracer B).

tent. The peak which corresponds to hGH is identified by comparison with the international standard of rec-hGH (t_R = 15.2 min). The central fraction of the peak, representing about 5% of the total peak volume, was shown to contain 16% of eluted rec-hGH with a specific activity of 64%. A portion of this material was labeled with ^{125}I, the tracer purification profile being presented in Fig. 2 and identified as "tracer A." Rec-hGH obtained from the same periplasmic extract after the usual six purification steps, when labeled, presented a similar purification profile (tracer B). As shown in Table 1 both tracers were obtained, as planned, with practically the same specific activity: 2.68 MBq/μg (72 μCi/μg) and 2.50 MBq/μg (68 μCi/μg), respectively, for tracers A and B. A high radioiodination yield was obtained for both tracers, being higher for tracer B. Distribution coefficient (K_D) values for both labeled preparations were practically identical and in perfect agreement with the cumulative statistics ($K_D = 0.445 \pm 0.029$, $n = 25$) described in previous work for ^{125}I-hGH (18).

Antiserum titration curves for both tracers are shown in Fig. 3. Tracer A, when compared with tracer B, presents significantly lower bindings at high antiserum concentrations; however, when using a RIA working dilution of 1:600,000, both tracers presented similar specific bindings: 46.1 and 50.7%, respectively. When, instead of the central fraction, a portion of the whole pool of rec-hGH was radioiodinated, a specific binding of 33.4% was obtained under the same conditions; however, in this pool rec-hGH was present at a specific activity of only 47%.

RIA standard curves carried out with tracers A and B were almost superimposable and provided agreement for hGH determination of three commercial-quality control levels evaluated in five independent assays (Table 2). Statistical analysis (t test) confirmed that there was no significant difference between hGH determinations using the two different tracers. Classical linear regression analysis comparing hGH concentrations obtained with the two different tracers provided a correlation coefficient (r) of 0.9997 and a slope of 1.023, indicating practically no bias.

The storage stability of the two tracers at −20°C consistently showed no significant difference (t test, $P > 0.05$) between their specific and nonspecific bindings over a period of up to 2 months (Fig. 4). The decrease in binding of 0.30%/day was somewhat higher than the 0.23%/day reported in previous work (18) for an analogous recombinant product. Since in that case ^{125}I-hGH was prepared at a much lower specific activity

TABLE 1

A Comparison between Typical Parameters Related to the Radioiodination of Rec-hGH from Osmotic Shock Fluid (Tracer A) and of Highly Purified Rec-hGH (Tracer B)

Preparation	Reacting protein (μg)	Reacting ^{125}I (MBq)	Labeling yield (%)	Specific activity (MBq/μg)	K_D of ^{125}I-hGH	Binding to Ab 1/600,000 (%)
Tracer A	3.8	15.5	65.7	2.68	0.447	46.1
Tracer B	10.0	32.0	78.0	2.50	0.449	50.7

TABLE 2

hGH Determination ($n = 5$ Assays, Using Intraassay Triplicate of Each Sample) of Commercial-Quality Control Samples, Utilizing ^{125}I-hGH Obtained from Osmotic Shock (Tracer A) and from Highly Purified Product (Tracer B)

Tracer	Low level (ng/mL)	Medium level (ng/mL)	High level (ng/mL)
A	3.61 ± 0.28	6.96 ± 0.40	11.47 ± 1.20
B	3.26 ± 0.29	6.37 ± 0.30	10.92 ± 1.15
t test ($P = 0.05$)	NS	NS	NS

(43.5 μCi/μg), the interlot reproducibility of our rec-hGH and the consistency of this stability test were verified by carrying out a second study. In this experiment, a tracer prepared with the same purified rec-hGH (lot 06297) labeled at a lower specific activity (50.5 μCi/μg) was compared to an internationally used preparation of pituitary hGH (NIDDK-I-3) labeled at a similar specific activity (45 μCi/μg). As shown in Fig. 5, the stability of ^{125}I-rec-hGH was, in this case, perfectly comparable with the previously reported value (18) and not significantly different from the stability of ^{125}I-pit-hGH (*t* test, $P > 0.05$), obtained by radioiodinating the preparation of hGH provided by the National Hormone and Pituitary Program (NIDDK-hGH-I-3).

DISCUSSION

The methodology described provides a novel, simple, and inexpensive procedure, capable of rapidly providing a good quality iodination grade protein for *in vitro* use. DNA recombinant techniques are now in use in many laboratories and transformed *E. coli* strains can be easily stored for long periods of time and even distributed to different laboratories. An overnight bacterial culture and a single-step purification process make it possible to always have freshly prepared protein that can be used for radioiodination or other purposes (e.g., internal reference, immunogen preparation). The instability of purified bioactive protein preparations, stored either in frozen solution or in the lyophilized state, is well known (25–30). The technique described here can circumvent this problem, providing better reagent continuity, improving intra- and interlaboratory reproducibility, and obviating the necessity to purchase expensive highly purified proteins.

Emphasis must also be given to the demonstrated accuracy and stability of the tracer obtained. These parameters were not affected at all by the lower purity of the product compared to the analogous preparation subjected to the regular six-step purification process. Finally, tracer stability was highly reproducible when compared to data from an equally radioactive preparation obtained and analyzed more than 5 years before (18). Moreover, these preparations of rec-hGH, when radioiodinated, have the same stability as a well-known and widely used international pituitary preparation that was submitted to the same labeling reaction.

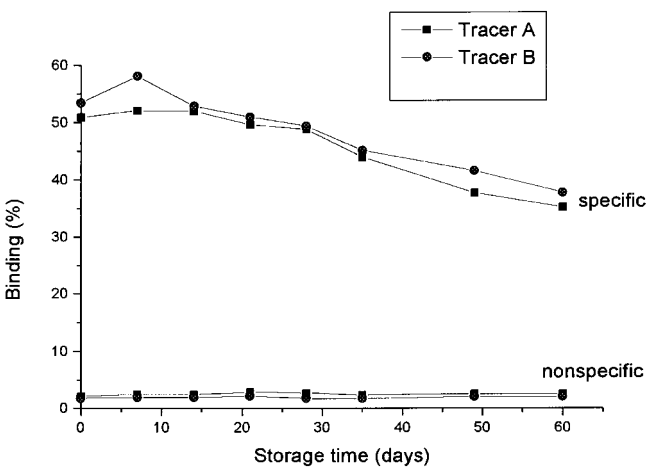

FIG. 4. Comparison of the stabilities of tracer A and tracer B during storage at −20°C over a 2-month period. Correlation curves (percentage of specific binding vs time): Tracer A slope, −0.305 ± 0.042 SD ($r = 0.948$, $n = 8$); Tracer B slope, −0.314 ± 0.039 SD ($r = 0.957$, $n = 8$).

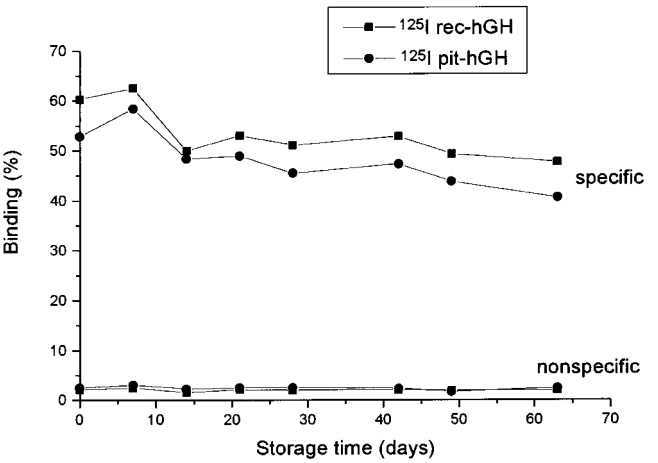

FIG. 5. Comparison of the stabilities of ^{125}I-pit-hGH and ^{125}I rec-hGH, both labeled at low specific activity, during storage at −20°C over a 2-month period. Correlation curves (percentage of specific binding vs time): ^{125}I-rec-hGH slope, −0.188 ± 0.063 SD ($r = 0.775$, $n = 8$); ^{125}I-pit-hGH slope, −0.216 ± 0.052 SD ($r = 0.861$, $n = 8$).

REFERENCES

1. Vahl, N., Jorgensen, J. O. L., Hansen, T. B., Klausen, I. B., Jurik, A-G, Hagen, C., and Christiansen, J. S. (1998) The favourable

effects of growth hormone (GH) substitution on hypercholesterolaemia in GH-deficient adults are not associated with concomitant reductions in adiposity: A 12 month placebo-controlled study. *Int. J. Obesity* **22,** 529–836.

2. Borges, M. F., Abelin, N. M. A., Menezes, F. O. M., Dahia, P. L. M., and Toledo, S. P. A. (1998) Calcitonin deficiency in early stages of chronic autoimmune thyroiditis. *Clin. Endocrinol.* **49,** 69–75.
3. Brooks, C. L., and Saiduddin, S. (1998) Phosphorylation of bovine prolactin eliminates luteotropic activity in the rat. *Life Sci.* **63,** 1281–1287.
4. Morganti, L., Soares, C. R. J., Affonso, R., Gout, P. W., Bartolini, P. (1998) Synthesis and characterization of recombinant, authentic human prolactin secreted into the periplasmic space of *Escherichia coli. Biotechnol. Appl. Biochem.* **27,** 63–70.
5. Steger, R. W., Chandrashekar, W. Z., Bartke, A., and Horseman, N. D. (1998) Neuroendocrine and reproductive functions in male mice with targeted disruption of the prolactin gene. *Endocrinology* **139,** 3691–3695.
6. Vonderhaar, B. K. (1998) Prolactin: The forgotten hormone of human breast cancer. *Pharmacol. Ther.* **79,** 169–178.
7. Fares, F. A., Yamabe, S., Ben-Menahem, D., Pixley, M., Hsueh, A. J. W., and Boime, I. (1998) Conversion of thyrotropin heterodimer to a biologically active single-chain. *Endocrinology* **139,** 2459–2464.
8. Miyai, K., Kumazawa, I., Saji, F., Azuma, C., Koyama, M., Kimura, T., Narizuka, Y., Kusunoki, M., and Murata, Y. (1998) Effect of site directed mutagenesis in the CMGCC region of the α-subunit on immunoreactive human thyrotropin. *Endocr. J.* **45,** 467–473.
9. Richard, F., Robert, P., Remy, J. J., Martinat, N., Bidart, J. M., Salesse, R., and Combarnous, Y. (1998) High-level secretion of biologically active recombinant porcine follicle-stimulating hormone by the methylotrophic yeast *Pichia pastoris. Biochem. Biophys. Res. Commun.* **245,** 847–852.
10. Rose, M. P., and Gaines-Das, R. E. (1998) Characterisation, calibration and comparison by international collaborative study of international standards for the calibration of therapeutic preparations of FSH. *J. Endocrinol.* **158,** 97–114.
11. Alsat, E., Guibourdenche, J., Couturier, A., and Evain-Brion, D. (1998) Physiological role of human placental growth hormone. *Mol. Cell. Biol.* **140,** 121–127.
12. Barreca, A., Bozzola, M., Cesarone, A., Steenbergh, P. H., Holthuizen, P. E., Severi, F., Giordano, G., and Minuto, F. (1998) Short stature associated with high circulating insulin-like growth factor (IGF)-binding protein-1 and low circulating IGF-II: Effect of growth hormone therapy. *J. Clin. Endocrinol. Metab.* **83,** 3534–3541.
13. Kerr, D. E., Liang, F., Bondioli, K. R., Zhao, H., Kreibich, G., Wall, R. J., and Sun, T. T. (1998) The bladder as a bioreactor: Urothelium production and secretion of growth hormone into urine. *Nature Biotechnol.* **16,** 75–79.
14. Wilson, M. E., (1998) Regulation of the growth hormone-insulin-like growth factor I axis in developing and adult monkeys is affected by estradiol replacement and supplementation with insulin-like growth factor I. *J. Clin. Endocrinol. Metab.* **83,** 2018–2028.
15. Kopchick, J., Chen, X. Z., Li, Y., Steger, R. W., Yun, J. S., Wagner, T. E., and Bartke, A. (1998) Differential in vivo activities of bovine growth hormone analogues. *Transgenic Res.* **7,** 61–71.
16. Mockridge, J. W., Aston, R., Morrell, D. J., and Holder, A. T. (1998) Cross-linked growth hormone dimers have enhanced biological activity. *Eur. J. Endocrinol.* **138,** 449–459.
17. Rudick,M. J. (1998) Controlled radioiodination of proteins *in* "Cell Biology—A Laboratory Handbook" (Celis, J. E., Ed.), Vol. 4, pp. 337–340, Academic Press, San Diego.
18. Ribela, M. T. C. P., Murata, Y., Morganti, L., Toniolo, D., and Bartolini, P. (1993) The use of recombinant human growth hormone for radioiodination and standard preparation in radioimmunoassay. *J. Immunol. Methods* **159,** 269–274.
19. Ribela, M. T. C. P., Bianco, A. C., and Bartolini, P. (1996) The use of recombinant human thyrotropin produced by Chinese hamster ovary cells for the preparation of immunoassay reagents. *J. Clin. Endocrinol. Metab.* **81,** 249–256.
20. Dalmora, S., Oliveira, J. E., Affonso, R., Gimbo, E., Ribela, M. T. C. P., and Bartolini, P. (1997) Analysis of recombinant human growth hormone directly in osmotic shock fluids. *J. Chromatogr. A* **782,** 199–210.
21. Jensen, E. B., and Carlsen, S. (1990) Production of recombinant human growth hormone in *Escherichia coli:* Expression of different precursors and physiological effects of glucose, acetate and salts. *Biotechnol. Bioeng.* **36,** 1–11.
22. Koshland, D., and Botstein, D. (1980) Secretion of beta-lactamase requires the carboxy end of the protein. *Cell* **20,** 749–760.
23. de Oliveira, J. E., Soares, C. R. J., Peroni, C. N., Gimbo, E., Camargo, I. M. C., Morganti, L., Bellini, M. H., Affonso, R., Arkaten, R. R., Bartolini, P., and Ribela, M. T. C. P. (1999) High-yield purification of biosynthetic human growth hormone secreted in *Escherichia coli* periplasmic space. *J. Chromatogr. A* **852,** 441–450.
24. Lowry D. B., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with Folin–phenol reagent. *J. Biol. Chem.* **193,** 265–275.
25. Nyberg, F., Bergman, P., Wide, L., and Roos, P. (1985) Stability studies on human pituitary prolactin. *Uppsala J. Med. Sci.* **90,** 265–277.
26. Becker, G. H., Tackitt, P. M., Bromer, W. W., Lefeber, D. S., and Rigghin, R. M. (1988) Isolation and characterization of a sulfoxide and a desamido derivative of biosynthetic human growth hormone. *Biotechnol Appl. Biochem.* **10,** 326–337.
27. Sizaret, P. (1988) The preparation of international reference materials for biological substances. *J. Biol. Stand.* **16,** 129–137.
28. Ressing, M. E., Jiskoot, W., Talsma, H., van Ingen, C. W., Beuvery, E. C., and Crommelin D. J. A., (1992) The influence of sucrose, dextran, and hydroooxypropyl-β-cyclodextrin as lyoprotectants for a freeze-dried mouse IgG_{2a} monoclonal antibody (MN 12). *Pharmaceut. Res.* **9,** 266–270.
29. Parlow, A. F.(1997) *in* "New, Super-Pure Human Pituitary Prolactin," technical sheet, NIDDK, Bethesda, MD.
30. Kaufmann, M. (1997) Unstable proteins: How to subject them to chromatographic separations for purification procedures. *J. Chromatogr. B* **699,** 347–369.