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

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Received April 8, 1999, and in revised form November 24, 1999

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 $\pm 50\%$ (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 $-20\,^{\circ}C$. 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.

Radioimmunoassay (RIA), 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

1 This investigation was supported by FAPESP, São Paulo, Brazil (Project Nos. 97/07400-0 and 99/01628-4), the Brazilian Research Council (CNPq) (Brasilia, Brazil), and the International Atomic Energy Agency (IAEA), Vienna, Austria (Technical Cooperation Project BRA 2/012).

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.
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 radiiodinated 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 radiiodinated 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, RR1 strain harboring an expression vector, constructed in our laboratory, in which the hGH gene (cDNA) was under control of the λ P1 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 Carl sen (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 A₆₀₀ 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 A₆₀₀ units was centrifuged at 4°C and 6000g 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).

Na¹²⁵¹ free of carriers and reductant was purchased from MDS Nordion (Canata, 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 Diagnostics (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 G2000SW column (60 cm × 7.5 mm i.d., particle size of 10 Å, and pore size of 125 Å) coupled to a 7.5 cm × 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

The¹²⁵¹I-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-
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 3000g 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 1:10⁵ to 1:2 × 10⁶.

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-
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 125I, 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/mg (72 μCi/μg) and 2.50 MBq/mg (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 ± 0.029, n = 25) described in previous work for 125I-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 125I-hGH was prepared at a much lower specific activity

### TABLE 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Reacting protein (μg)</th>
<th>Reacting 125I (MBq)</th>
<th>Labeling yield (%)</th>
<th>Specific activity (MBq/μg)</th>
<th>K_D of 125I-hGH</th>
<th>Binding to Ab 1/600,000 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracer A</td>
<td>3.8</td>
<td>15.5</td>
<td>65.7</td>
<td>2.68</td>
<td>0.447</td>
<td>46.1</td>
</tr>
<tr>
<td>Tracer B</td>
<td>10.0</td>
<td>32.0</td>
<td>78.0</td>
<td>2.50</td>
<td>0.449</td>
<td>50.7</td>
</tr>
</tbody>
</table>
(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 125I-rec-hGH was, in this case, perfectly comparable with the previously reported value (18) and not significantly different from the stability of 125I-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.

**TABLE 2**

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Low level (ng/mL)</th>
<th>Medium level (ng/mL)</th>
<th>High level (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.61 ± 0.28</td>
<td>6.96 ± 0.40</td>
<td>11.47 ± 1.20</td>
</tr>
<tr>
<td>B</td>
<td>3.26 ± 0.29</td>
<td>6.37 ± 0.30</td>
<td>10.92 ± 1.15</td>
</tr>
<tr>
<td>t test (P = 0.05)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

**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 125I-pit-hGH and 125I 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): 125I-rec-hGH slope, −0.168 ± 0.063 SD (r = 0.775, n = 8); 125I-pit-hGH slope, −0.216 ± 0.052 SD (r = 0.861, n = 8).

**REFERENCES**


