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Ultraviolet Scanning Densitometry for Detection, Quantitation, and Preparative Elution of Protein Bands from Unstained Gels[1]

Paolo Bartolini, Rosangela Arkaten, and Maria Teresa Ribela

Division of Medicine, Department of Application of Nuclear Techniques in Biological Sciences, National Nuclear Energy Commission (IPEN-CNEN), Cidade Universitaria, 05508 São Paulo, Brazil

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Ultraviolet scanning of gel rods was used to identify and quantify protein bands in a nondestructive manner with good precision and sensitivity. This same technique, applied on a preparative scale, allowed quantitative protein elution, by reversed electrophoresis, from gel slices completely sealed in a dialysis bag. Protein recovery approached the theoretical yield (93.5 ± 5%), with practically no interfering substances, and the entire preparative process (first electrophoresis, densitometric scanning, and reversed electrophoresis) could be performed in approximately 6 h. Its application to human growth hormone has shown no alteration in the biological activity of this protein. © 1989 Academic Press, Inc.

Many authors have described the extraction or continuous elution of proteins in preparative polyacrylamide gel electrophoresis (PAGE),[2] using quite different techniques (1–19). Some of them use unstained gels in which the biological properties of the sample are not altered, taking advantage of ultraviolet visual localization (1,7,9,14,17), spectrophotometric analysis (10,15), or direct uv scanning densitometry (4,6,11,12). Some authors obtain quantitative recoveries using stained or dansylated proteins (5,9,13,16), but most of them accept yields of the order of 50–70%. The reported methods often use expensive and complicated machineries or time-consuming multiple-step techniques, sometimes combining PAGE, gel filtration, adsorption, and ionic exchange chromatography (1,3,5,8,11,15,19,20). Of particular interest are the studies of Kapadia and Chrambach (2) and Nguyen *et al.* (15), from the same research group, who carried out extensive studies on the nature of protein losses using quite different techniques and reaching fairly comparable conclusions and recoveries: 50–70% and 76–80%, respectively. Lack of purity, in addition to poor yields, has also been one of the main problems, since interferences due to the presence of acrylates was repeatedly mentioned by several authors (3,5,6,11,15, 20,21).

We present here a simple, single technique, a rapid and efficient preparative method for the quantitative recovery of milligram amounts of proteins from polyacrylamide gels. This technique utilizes reverse-electrophoretic elution after direct ultraviolet densitometric scanning. This is made possible by the development of an accurate quantitative analytical technique, based on this same nondestructive densitometry and carried out on a smaller gel rod.

The analytical and preparative techniques described here are exemplified by extraction and recoveries of different amounts of two standard proteins and of a polypeptide hormone, a type of compound for which the method should have particularly interesting applications since, as it is shown, it fully preserves its biological activity.

MATERIALS AND METHODS

Electrophoretic grade acrylamide was obtained from Interlab (São Paulo, Brazil), *N,N'*-methylenebisacrylamide was from Merck-Quimitra (São Paulo, Brazil), and Temed was from Bio Rad-Erviegas (São Paulo, Brazil). $Na^{125}I$, carrier and reductant free, at a specific activity of 350 mCi/ml, was purchased from New England Nuclear-

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[2] Abbreviations used: PAGE, polyacrylamide gel electrophoresis; Temed, *N,N,N',N'*-tetramethylethylenediamine; BSA, bovine serum albumin; STI, soybean trypsin inhibitor; hGH, human growth hormone.

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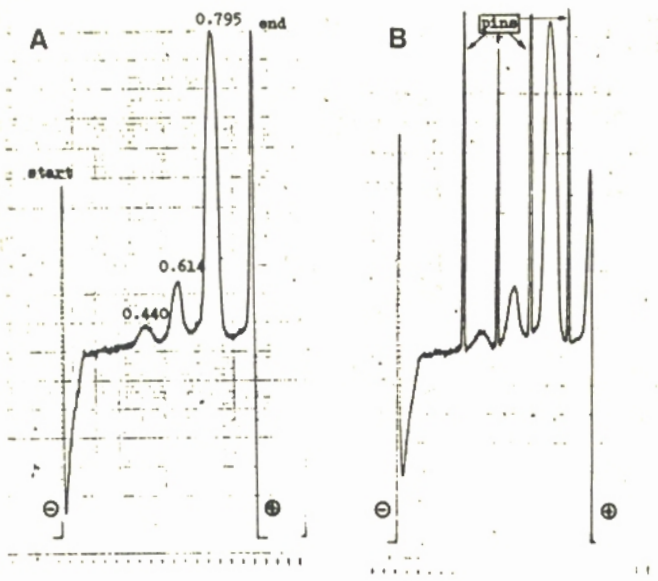

FIG. 1. Preparative scanning densitometry of BSA (2-mg load) on a large gel (length, 11 cm; diameter, 10 mm). (A) First reading. (B) Second reading, after the protein bands are delimited with steel pins. The figures indicate the electrophoretic rates of migration (R_m). Gel concentration, 7%.

Dupont (Boston, MA). Bovine serum albumin (BSA), radioimmunoassay grade, and soybean trypsin inhibitor (STI) were from Sigma Chemical Co. (St. Louis, MO). Growth hormone (hGH-IPEN) was extracted and purified from human pituitaries in this laboratory as previously described (22) or kindly provided (hGH-NIADDK) by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (Baltimore, MD).

The 7% PAGE technique was performed as described elsewhere (22), ammonium persulfate being completely substituted by riboflavin according to a modification of the method of Davis (23).

Analytical Scanning Densitometry

For this purpose the PAGE technique was carried out in glass tubes 11 cm long and with an internal diameter of 5 mm. After removal from the tube, the gel rod was placed horizontally in a test tube filled with distilled water for about 5 min in order to decrease the influence of the interfering substances that are rapidly washed out. The gel was then scanned inside the 10 × 15 × 120-mm quartz cuvette, almost completely filled with water, with a high resolution scanning densitometer (Joyce Loebl, Gateshead, UK), whose fixed ultraviolet wavelength range is 220–310 nm.

Preparative Scanning Densitometry

In this case, a larger glass tube (11-cm length, but with a 10-mm i.d.) was used. The protein sample (loads up to 2 mg) was dissolved in 1 ml of 0.01 M $NaHCO_3$, with an addition of 200 μl of 40% sucrose solution. Electrophoresis was run for about 3 h at 5 mA and 200 V. The densitometric reading is carried out twice; the second time the position of the protein band that has to be eluted is marked using steel pins whose diameter is about 0.5 mm, as exemplified in Fig. 1 for the case of BSA.

Protein Elution by Reversed-Electrophoresis

The gel segment containing the protein band to be eluted (1.5-cm maximum length) was cut at the two delimiting pins and sealed, together with 2 ml of running buffer, inside a dialysis sack knotted at both extremities (Fig. 2). A supporting gel was prepared by introducing the polymerization mixture with a syringe and two plastic tubings as shown in the figure, taking care to eliminate all air bubbles. Light-induced polymerization was performed at 4°C, protecting the protein-containing gel segment with a cap of aluminum foil. Reversed-electrophoresis (i.e., with the anode in the upper reservoir) was run at 10 mA and 200 V for ~2 h, optimizing this elution time according to the migration rate of each particular band. The protein recoveries were determined via analytical scanning densitometry; in some cases, the purity of the extracted protein was also controlled by weight, after dialysis and lyophilization, applying a criterion similar to that used by Nguyen *et al.* (15).

hGH Biological Assay by the Weight Gain of Hypophysectomized Rats

The 2 × 2 factorial assay design was carried out, over a 10-day period, as described in previous work (24), using four groups of 10 rats each, for two doses (10 and 20 μg/

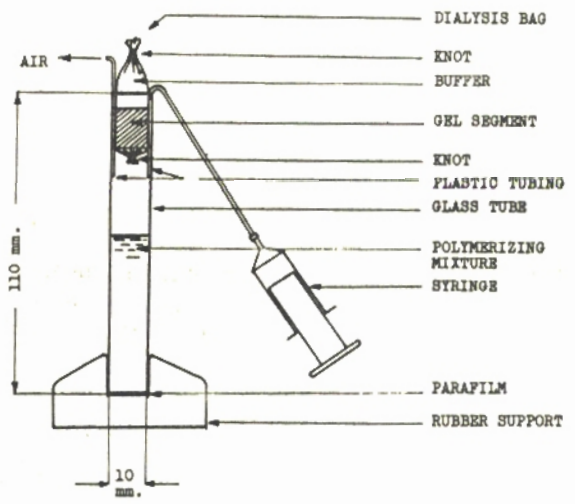

FIG. 2. Scheme of the electrophoretic system for the protein elution from gel slices.

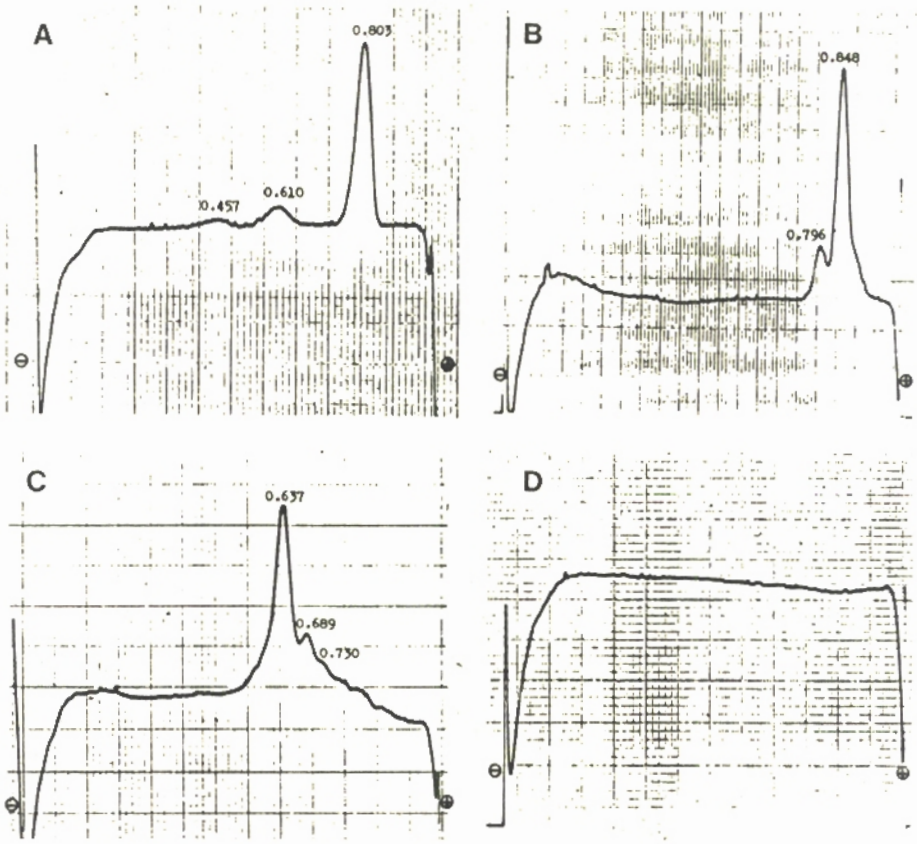

FIG. 3. Examples of analytical scanning densitometry, with the corresponding R_m, for 100 μg of (A) BSA, (B) STI, (C) hGH. (D) Blank. Gel concentrations, 7% for BSA and hGH; 8% for STI.

rat/day) and two preparations: control hGH (S) and PAGE-eluted hGH (U).

RESULTS

The method described was applied to the purification of two commonly used standard proteins (BSA and STI) and a polypeptide hormone (hGH). Before the preparative gel was run, each protein was analyzed from a qualitative and quantitative point of view (Fig. 3), in order to choose the best electrophoretic conditions and set up a quantitative calibration curve, as exemplified for BSA in Fig. 4. The calibration curves for the three different proteins, in the range 5–100 μg, were the following:

1. BSA $Y = 4.587X + 0.020$ $(n = 21; r = 0.9999)$
2. STI $Y = 5.667X - 3.381$ $(n = 11; r = 0.9982)$
3. hGH $Y = 4.493X + 7.057$ $(n = 9; \ r = 0.9994)$,

where Y indicates the peak area (mm^2), X the applied dose (μg), r the correlation coefficient, and n the number of determinations ($P < 0.001$ in all cases).

The sensitivity of the quantitative technique, calculated via a t test (one sided, $P = 0.05$) using replicate determinations of the zero point (control gel) and the lowest significant dose, presented values of 1.5–1.7 μg of protein.

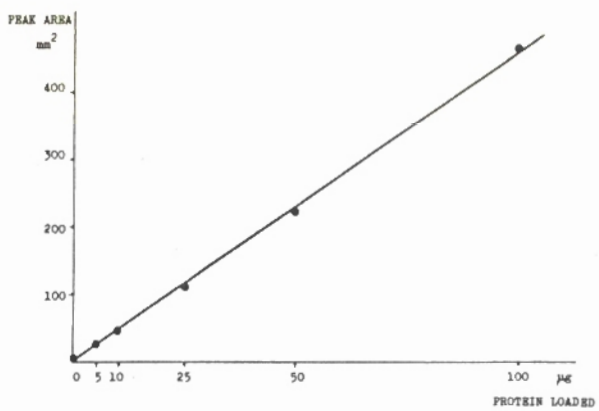

FIG. 4. Quantitative uv scanning densitometry: calibration curve for BSA in 7% acrylamide gel. Peak areas, in square millimeters, were calculated manually.

TABLE 1

Recovery of Proteins Eluted from Gel Slices

Protein	Amount applied (μg)	Recovery (%)
Bovine serum albumin	500	95.7
Bovine serum albumin	1000	93.8
Bovine serum albumin	2000	96.5
Soybean trypsin inhibitor	500	95.6
Soybean trypsin inhibitor	1000	95.9
Soybean trypsin inhibitor	2000	97.3
Human growth hormone	500	92.4
Human growth hormone	2000	94.7

TABLE 2

Yield and Recovery of Lyophilized BSA Samples

Exp. No.	Lyophilized volume (ml)	Recovery based on weight (mg)	Recovery based on $A_{220\text{-}310}$ (mg)	Protein recovery (%) based on $A_{220\text{-}310}$/weight
1	1.78	1.30	1.34	103.1
2	1.67	1.40	1.36	97.1
3	1.35	1.05	1.07	101.9

As can be seen in Fig. 3, the three preparations we used are heterogeneous. We know from previous experiments that BSA is composed of three mass isomers (monomer, dimer, and trimer), STI of two charge isomers, and our hGH preparation of two or more isohormones, including deamidated or proteolitically cleaved forms (24).

The final recoveries obtained for different loads of the three proteins are presented in Table 1. These were obtained via the present analytical scanning densitometry technique, running a standard preparation of the specific protein being eluted, each time, to check the validity of the predetermined standard curve.

In the case of BSA, recoveries refer to the monomeric form (for this preparation 82% of the total), which was obtained completely free from its polymeric forms (Fig. 5), while, in the case of STI and hGH, the various isomers were eluted together. Due to its high migration rate, the best electrophoretic conditions for STI elution were found to be 8% rather than 7% acrylamide concentration. In the case of the highest protein load (2 mg) of BSA, the eluted sample was also dialyzed and lyophilized, the amount of eluted material being also determined by weight, thus allowing an evaluation of the resulting protein content and possible acrylates contamination (Table 2).

The 2 × 2 factorial bioassay data relative to PAGE-eluted hGH are presented in Table 3. Through an analysis of variance we can observe that the difference between preparations is not significant and neither is the slope divergence of the dose–response curves, confirming that there has not been a quantitative or qualitative alteration. The index of precision, quite low for an "*in vivo*" bioassay, also confirms the good quality of this test.

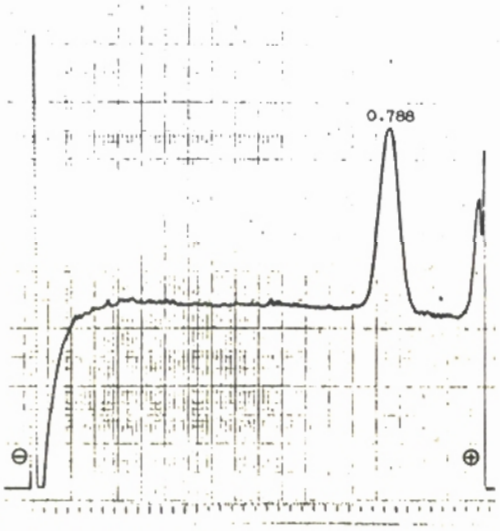

FIG. 5. Example of a purified BSA monomeric preparation obtained after preparative scanning densitometry and protein elution by reversed electrophoresis.

DISCUSSION

The proposed procedure is based on a quantitative analytical method used in conjunction with a preparative gel electrophoresis. Both techniques employ densitometric ultraviolet scanning of unstained protein bands, a procedure which is particularly suitable for the purification of biologically active polypeptides.

The analytical scanning densitometry described here is extremely simple and rapid, does not require the expensive quartz tubes, columns, or apparatus often used (4,21,25–27), and, in particular, has proven to provide clean and homogeneous electrophoretograms, with very good baseline stability, as can be seen in the accompanying figures. These qualities are, in fact, very seldom observed in works which report uv scanning densitometry of unstained protein bands. We found that the complete substitution of ammonium persulfate with riboflavin in the gel polymerization and the soaking of the gels in water for some minutes before scanning were important factors for avoiding nonuniform distribution of uv-absorbing materials. This has, in fact, been described as

TABLE 3

hGH Biological Assay: Weight Gain of Hypophysectomized Rats; 2 × 2 Factorial Assay Design

Doses: 10 (1) and 20 μg/rat/day (2)
Preparations: Control hGH (S) and PAGE-eluted hGH (U)
Animals: Four groups of 10 rats each ($N = 40$)

Doses (μg/day)	Preparations	Individual weight increase (g/10 days) 1	2	3	4	5	6	7	8	9	10	Total (g/10 days)
10	U1	9.5	7.5	8.0	7.5	10.5	10.0	7.5	10.0	7.3	11.0	88.8
10	S1	11.7	9.5	7.5	8.0	11.0	6.0	8.5	10.0	8.5	10.4	91.1
20	U2	11.5	12.0	13.5	12.0	13.0	11.5	11.5	13.4	12.0	12.0	122.4
20	S2	13.5	13.3	14.0	10.5	13.4	11.3	12.5	11.5	12.5	14.5	127.0
												S(y): 429.3

Results

	Significance test ($P = 0.05$)
Difference between preparations	Not significant
Difference between doses	Significant
Slope divergence	Not significant
Difference between groups	Not significant
Potency (U-activity/S-activity)	0.93
Combined slope	11.54
Standard deviation	1.15
Index of precision (λ)	0.100
Fiducial limits	0.80 (lower) 1.08 (upper)

one of the main limiting factors related to the application of this densitometric technique (27).

The sensitivity obtained is at least as good as that reported by other authors for quantitative measurements of unstained protein bands (12,21,25–27). The calibration curves determined here for the three proteins analyzed, which are seldom presented in literature with their statistical parameters (28), show a very good linearity and precision in the useful operative range of 5–100 μg of applied protein.

The preparative technique is simple, inexpensive, and rapid, allowing the whole process to be carried out in about 6 h. It presents the advantage of using a single, very flexible technique (PAGE), in contrast to other methods which require combinations of different techniques. When compared to other techniques based on ultraviolet scanning, the present method is the only one we know in which recoveries approached theoretical yields. This must be emphasized as one of its major advantages, especially considering its application to the purification of precious polypeptide hormones.

The high purity of the eluted protein must also be emphasized, considering the often-mentioned interferences due to the presence of polyacrylates. Their presence, in fact, did not have any ponderable significance, as we could easily demonstrate by eluting a blank under the same conditions. On the contrary, large amounts of contaminants could be isolated when, in preliminary experiments, the gel segment was submitted directly to overnight buffer extraction.

The PAGE technique is highly flexible and, as is well known, the resolution of mass or charge isomers can be obtained with the use of longer gels, by changing the acrylamide concentration, pH, or ionic strength of the polymerization or running buffer, or by rerunning the segment derived from the first electrophoresis on a new gel. In the case of hGH and STI, the various charge isomers were intentionally not separated, our main interest being complete recoveries with, in the former case, retention of biological activity. Nonetheless, with the described technique, isolation of the different peaks is possible, although outside the scope of the present work.

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