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ABSTRACT

We present here a technique for the purification of proteins carried out by a quantitative analytical method used in conjunction with a preparative gel electrophoresis. Both methods employ desitometric ultraviolet scanning of unstained protein bands, a procedure which is particulary suitable for the purification and recovery of biologically active polypeptides. In short, the purifield extracted protein, isolated in a segment cut out from a preparative gel, is recovered by a second (reversed) electrophoresis. We performed the extractions and recoveries of different amounts of two standard proteins (BSA and STI) and a polypeptide hormone (hGH). Our main interest, especially for the hormone is the complete protein recovery with retention of bio and immunoactivity and high purity. For the proteins tested, the mean recovery was of 93 ± 5% obtaining a mean purity of 95 ± 7%. We conclude that the proposed method should have interesting applications, particulary in the obtention of very pure hormones, as are needed for radioligand assays, for radiolabelling and specific antibody raising. We emphasize the simplicity and rapidity of the method (the entire preparative process: first electrophoresis, UV scanning and reversed electrophoresis can be performed in approximately six hours) and its efficiency in recovering pure proteins even on a milligram scale. We thank the support from the IAEA (4299/RB) and FINEP (43.86.0351.00) and CENE (Brazil).

RECUPERAÇÃO DE PROTEÍNAS DE ALTA PUREZA DE GEIS DE POLIACRILAMIDA USANDO DESITOMETRIA ULTRAVIOLETA

RESUMO

Nós apresentamos aqui uma técnica para a purificação de proteínas realizada por um método analítico quantitativo usado em conjunto com um gel preparativo. Ambos os métodos empregam leitura densitométrica ultravioleta das bandas proteicas não coradas, um procedimento que é particularmente u-

til para a purificação e recuperação dos polipeptideos biologicamente ativos. Em resumo, a proteína extraída purificada, isolada num segmento cortado do gel preparativo é recuperada por uma segunda eletroforese (reversa). Nós realizamos as extrações e recuperações de diferentes quantidades de duas proteínas padrão (BSA e STI) e de um hormônio polipeptídeco (hGH). Nosso principal interesse, especialmente para o hormônio, é a recuperação total da proteína com retenção da bio e imunoatividade e alta pureza. Para as proteínas testadas a recuperação média foi de 93 ± 5% obtendo-se uma pureza média de 95 ± 7%. Nós concluimos que o método proposto tem aplicações interessantes particularmente na obtenção de hormônios muito puros como são necessários para ensaios radioligantes, para serem marcados e para produção de anticorpos. Enfatizamos a simplicidade e rapidez do método (o processo inteiro: primeira eletroforese, densitometria UV e eletroforese reversa podem ser realizadas em aproximadamente 6 horas) e sua eficiência na recuperação de proteínas puras em escala de miligramas.

INTRODUCTION

The extraction of proteins in preparative polyacrylamide gel electrophoresis (PAGE) has been described by many authors, but only some of them have used direct UV scanning densitometry (1-4). None of them ever presented quantitative recoveries, accepting yields of the order of 50-80 % (5-6).

We present here a simple, single step technique which serves as 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 non-destructive densitometry and carried out 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. A radioiodinated tracer protein was used to identify protein losses and, consequently, to improve the general recoveries.

MATERIALS AND METHODS

Electrophoretc grade acrylamide was obtained form Interlab (São Paulo, Brazil), N,N' -- methylenebisacrylamide was from Merck-Quimitra (São Paulo, Brazil) and TEMED from Bio Rad-Erviegas (São Paulo, Brazil). $Na^{125}I$, carrier and reductant free, at a specific activity 350 mCi/ml, was purcha - sed from New England Nuclear-Dupont (Boston, USA).Bovine serum albumin(BSA) Radioimmunoassay grade and Soybean trypsin inhibitor (STI) were from Sigma Chem. Co. (St. Louis, USA). Growth hormone (hGH-IPEN) was extracted and purified from human pituitaries in this laboratory as previously described (7) or kindly provided (hGH-NIDDK) by the National Institute of Diabetes , Digestive and Kidney Diseases (Baltimore, USA).

The 7% PAGE technique was performed as described elsewhere (7), ammonium persulfate being completely substituted by riboflavin according to a modification of the method of Davis (8).

Analytical Scanning Densitometry

For this purpose the PAGE technique was carried cut in 5 ϕ x 110 mm glass tubes. After removal from the tube using a long needle syringe with constant injection of distilled water, the gel rod was placed horizontally in a test tube filled with distilled water for about five minutes in order to decrease the influence of the interfering substances that are rapidly washed out. The gel was then scanned inside the 10 x 15 x 120 mm quartz cuvette, almost completely filled with water, of a Joyce Loebl Scan 400 high resolution scanning densitometer (Gateshead, UK), whose fixed ultraviolet wavelength range is 220 - 310 nm.

Preparative Scanning Densitometry

In this case, a larger glass tube (10 ϕ x 110 mm) was used. The protein sample (loads up to 2 mg) was dissolved in 1 ml of 0.01 M $NaHCO_3$, with addition of 200 µl of a 40% sucrose solution. Electrophoresis was run for about 3 hours at 5 mA and 200 V. The densitometric reading is carried out twice; the second time, the protein band that has to be eluted is delimited using steel pins, 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 with a razor blade at the two delimiting pins and sealed, together with 2 ml of running buffer, inside a dialysis sack knotted at both extremities (Fig. 2). The upper knot should done with a cotton thread such as to eliminate the air completely. The sack was then introduced into the top of a second glass of the same diameter, leaving the upper por -

tion, with part of the 2 ml buffer, out of the tube. A supporting gel was prepared by introducing the polymerization mixture with syringe and two plastic tubings as shown in Fig. 2, 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. The gel tube was then introduced into be preparative cell, where the electric poles had been reversed (i.e. with the anode in the upper reservoir). Electrophoresis was run at 10mA and 200 V for ~2 hours, optimizing this elution time according to the migration rate of each particular band. After electrophoresis, the sack was carefully cut at the top and the buffer containing the eluted protein pipetted, washing the gel segment with fresh buffer before discarding it. The protein recoveries were determined via analytical scanning densitometry; in some cases, the purity of the extracted component was also controlled by weight, after dialysis and lyophilization.

RESULTS

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

1. BSA $Y = 4.587 X + 0.020$ ($n=21$ $r = 0.9999$)
2. hGH $Y = 4.493 X + 7.057$ ($n= 9$ $r = 0.9994$)

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 both cases).

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

The final recoveries obtained for three 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 pre-determined standard curve. Such high yields were obtained after a detailed study of protein losses along the whole process, carried out with ^{125}I-BSA.

This tracer study had indicated 97.3 ± 5.8% recoveries after the preparative gel, 71.3 ± 4.9% after reversed electrophoresis and 49.8 ± 4.0% after the final analytical step. Considering that the major sources of protein losses were the last two steps, the following modifications were introduced:

a) The volume of the elution buffer inside the dialysis sack was increased from 1 to 2 ml;
b) dialysis before the analytical step to eliminate interferences due to the glycine phosphate buffer was replaced by a simple sample dilution;
c) longer analytical gels (11 instead of 8 cm), three dosages and a higher amplification in the densitometric reading were used.

In the case of BSA, recoveries refer to be monomeric form (for this preparation 82% of the total), which was obtained completely free from its polymeric forms (Fig. 4), while, in the case of STI and hGH, the various charge 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), the eluted sample was also dialyzed and lyophilized; the protein content was determined by weight, thus obtaining an evaluation of the resulting purity and possible acrylate contamination (Table 2).

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 here described is extremely simple and rapid, does not require the expensive quartz tubes, columns or apparatus often used (1, 9-12) and, in particular, has proven to provide clean and homogeneous electrophoretograms, as can be seen in the accompanying figures.

The obtained sensitivity is at least as good as that reported by other authors for quantitative measurements of unstained protein bands (1 , 4, 9-12).

The preparative technique is simple, inexpensive and rapid, allowing the whole process to be carried out in about six hours. It presents the advantage of using a single, very flexible technique (PAGE, in contract to

other methods which require combinations of different techniques.

The high purity of the eluted protein must also be emphasized, espe - cially considering the often mentioned interferences due to the presence of polyacrylates.

TABLE 1

RECOVERY OF PROTEINS ELUTED FROM GEL SLICES

Protein	Amount applied (µg)	Recovery (%)
Bovine serum albumin	500	95.7
"	1000	93.8
"	2000	96.5
Soybean trypsin inhibitor	2000	97.3
Myoglobin	2000	94.3
Human growth hormone	2000	94.7

TABLE 2

Yield and Purity of Lyophilized BSA samples

Exp. nº	Lyophilized Volume (ml)	Recovery based on weight (mg)	Recovery based on $A_{220-310}$ (mg)	Purity based on $\frac{A_{220-310}}{\text{weight}}$ (%)
1	1.25	0.70	0.78	111.4
2	1.78	1.30	1.34	103.1
3	1.67	1.40	1.36	97.1
4	1.35	1.05	1.07	101.9

FIG 1

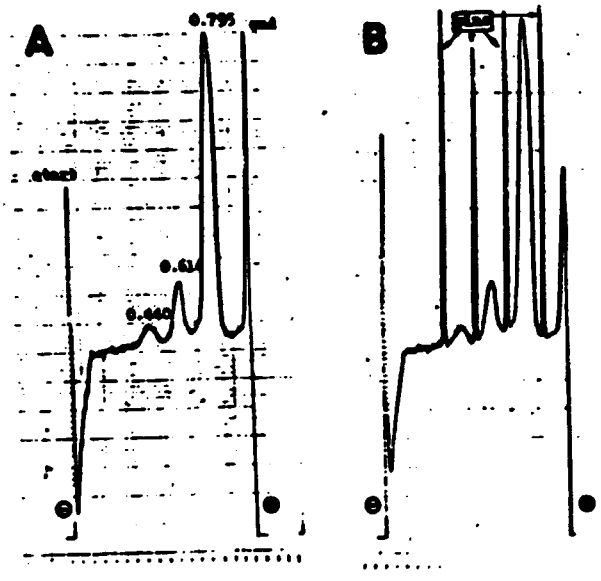

A
B

FIG 2

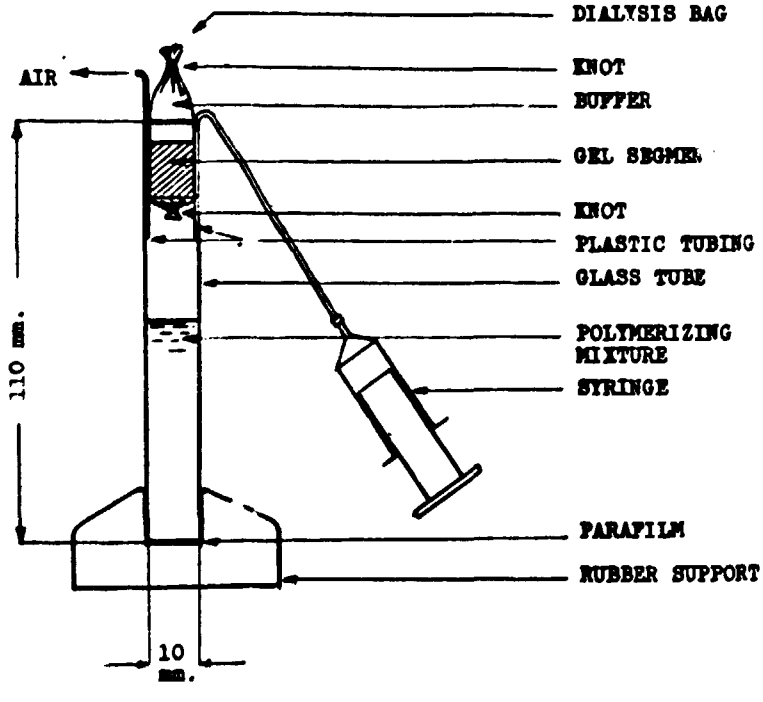

DIALYSIS BAG
AIR
KNOT
BUFFER
GEL SEGMEN
KNOT
PLASTIC TUBING
GLASS TUBE
110 MM.
POLYMERIZING MIXTURE
SYRINGE
PARAFILM
RUBBER SUPPORT
10 MM.

FIG 3.

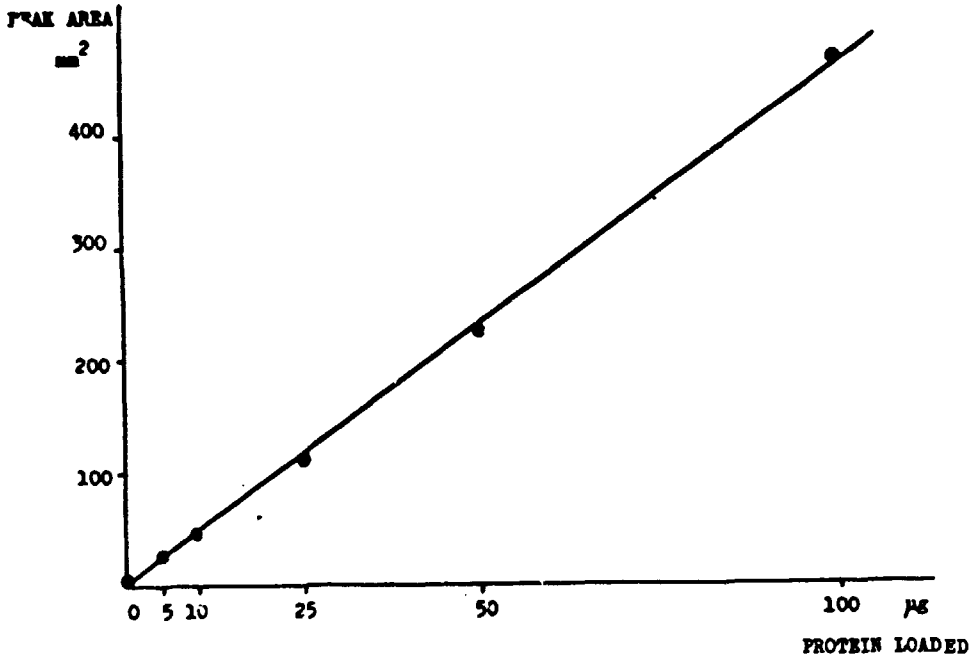
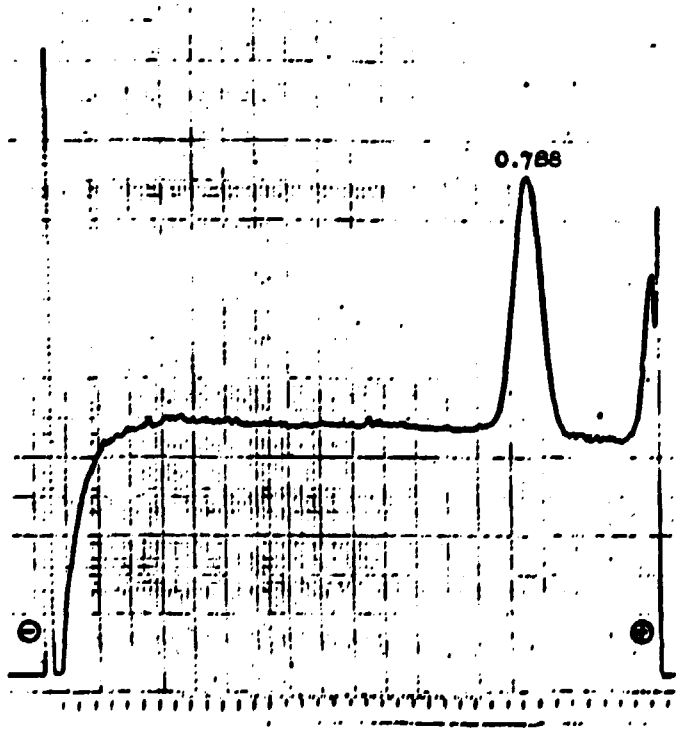

PEAK AREA
mm²
400
300
200
100
0
5
10
25
50
100
µg
PROTEIN LOADED

FIG4


0.788

LEGENDS

Fig. 1. Preparative scanning densitometry of BSA (2 mg load) on a large gel (10 ϕ x 110 mm). (A) First reading, (B) Second reading, after delimiting the protein bands with steel pins. The figures indicate the electrophoretic rates of migration (Rm).

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

Fig. 3. Quantitative UV scanning densitometry calibration curve for BSA in 7% acrylamide gel. Peak areas, in mm^2, are calculated manually.

Fig. 4. Example of a purified BSA monomeric preparation obtained after preparative scanning densitometry and protein elution by reversed-electrophoresis.

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