PROTEIN TRANSFER TO NITROCELLULOSE FILTERS

A simple method for quantitation of single proteins in complex mixtures

R. T. M. J. VAESSEN, J. KREIKE+ and G. S. P. GROOT*

Section for Molecular Biology, Laboratory of Biochemistry, University of Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands

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1. Introduction

In the course of our study of the biogenesis of the cytochrome bc_1 complex (complex III) of yeast mitochondria we became interested in the steady state concentrations of the different subunits of this complex.

Several procedures have been described to transfer proteins from polyacrylamide gels to diazobenzyloxymethyl-cellulose (DBM) paper [1–4] or nitrocellulose filters [4–6] electrophoretically or by diffusion. In principle these techniques offer a simple method for measuring the concentrations of single proteins in complex mixtures like whole cell lysates provided that a suitable immunological detection method is available. However, exact data about the transfer efficiency of proteins from gels containing sodium dodecyl sulphate (SDS) to nitrocellulose filters by electrophoresis and the reliability of the immunodetection method are scarce. We have studied the quantitative aspects of the protein blotting technique in order to estimate the amounts of individual complex III subunits in cell lysates.

The procedure used for transfer is a modification of the one originally described in [6]. No special apparatus is required apart from two perforated stainless steel plates which are used as electrodes.

Here, we report that under our conditions the transfer efficiency of a given protein from a SDS–polyacrylamide gel to nitrocellulose filters depends on its M_r-value. After immunodetection with specific antisera and ^{125}I-labelled protein A, the amount of label bound to each antigen increases linearly with the amount of protein applied to the gel. The ^{125}I-labelled protein A bound to the filter is not removed during successive incubations with different antisera.

2. Materials and methods

Yeast (*Saccharomyces carlsbergensis* NCYC74) was grown on 1% yeast extract–15% glucose or on a semi-synthetic medium containing sodium lactate as carbon source, as in [7]. Whole cell lysates were prepared by shaking yeast cells with glass beads [8] and removal of unbroken cells and large debris by centrifugation at 1500 × *g* for 5 min. The cytochrome bc_1 complex was prepared as in [9]. *Staphylococcus aureus* protein A (Pharmacia) and cytochrome bc_1 complex were iodinated with [^{125}I]iodide according to [10]. Electrophoretic procedures using SDS–polyacrylamide gels have been described [11].

Proteins were transferred to nitrocellulose filters (Sartorius; 0.45 μm pore size) using the set-up shown in fig.1 at 60 V for 2 h using 192 mM glycine–25 mM Tris–20% methanol (pH 8.3) as buffer [6]. Special care was taken to avoid all air bubbles between the layers. After the transfer, filters were incubated in phosphate-buffered saline (10 mM NaH_2PO_4 (pH 7.2), 150 mM NaCl)–8% bovine serum albumin to saturate remaining protein-binding sites for 30–60 min at 40°C [6]. The antiserum reaction was done for 16 h at 37°C at 1:50 diluted serum in 5–10 ml phosphate-

+ Present address: Institut für Allgemeine Biochemie der Universität Wien, Währinger Strasse 38, A-1090 Wien, Austria

* Present address: Laboratory of Biochemistry, Free University, de Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

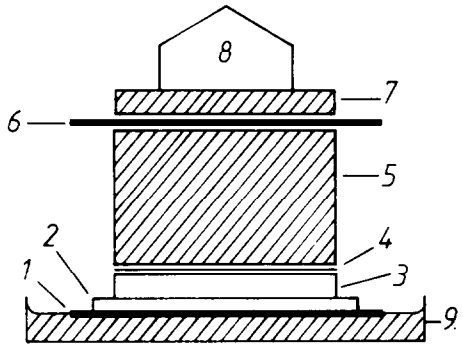

Fig.1. Electrophoretic transfer of proteins from SDS–polyacrylamide gels to nitrocellulose filters. The apparatus is assembled as follows: the cathode (1) is placed in a tray and on top of it are successively spread a sheet of Schleicher and Schüll paper (no. 2294) soaked in 0.5% SDS (2), the gel (3), a sheet of nitrocellulose filter (4) and 10–15 Schleicher and Schüll paper sheets soaked in the buffer (5), described in section 2. On the anode (6), 3–5 sheets soaked in the same buffer (7) are spread with a weight (1.5 kg) on top of them to maintain proper contact (8). Finally, buffer is added (9). Care must be taken to avoid all air bubbles between layers. Two stainless steel plates (150 × 200 × 1 mm) with perforations of 5 × 5 mm are used as electrodes. Transfer is done at room temperature for 2 h. The voltage is gradually increased to 60 V, assuring the current does not exceed 250 mA.

buffered saline–8% bovine serum albumin with vigorous shaking. Unbound antiserum was washed away with phosphate-buffered saline (3 × 5 min) and the filter was incubated in the same volume of phosphate-buffered saline–8% bovine serum albumin with 10^6 cpm ^{125}I-labelled protein A for 1–2 h at room temperature with vigorous shaking. Filters were washed in phosphate-buffered saline–1% Triton X-100 for 2–3 h to remove unbound protein A, dried and autoradiographed on pre-fogged Kodak XR-1 film at −70°C (usually 2–16 h) using Ilford intensifying screens.

Autoradiograms were scanned at 550 nm in a Gilford 2400-2 spectrophotometer equipped with a linear transport system. In some cases the radioactive bands were cut out and counted in a Packard 5230 auto-gamma scintillation spectrophotometer. Protein was determined according to [12].

3. Results and discussion

To determine the efficiency of transfer of proteins from SDS–polyacrylamide gels, samples of ^{125}I-labelled cytochrome bc_1 complex were electrophoresed on an 11% slab gel and subsequently transferred to nitrocellulose filters as in section 2 and fig.1. The results are given in fig.2. Comparison with the autoradiogram of the original gel (lane 1) shows that the nitrocellulose filter (lane 2A) is a faithful replica within certain limits. Two observations deserve comment:

(i) Proteins of low M_r (<25 000) appear to be transferred more completely in 2 h than proteins with higher M_r-values (lane 2B).

(ii) Not all the protein transferred electrophoretically is retained on the filter as is apparent from the

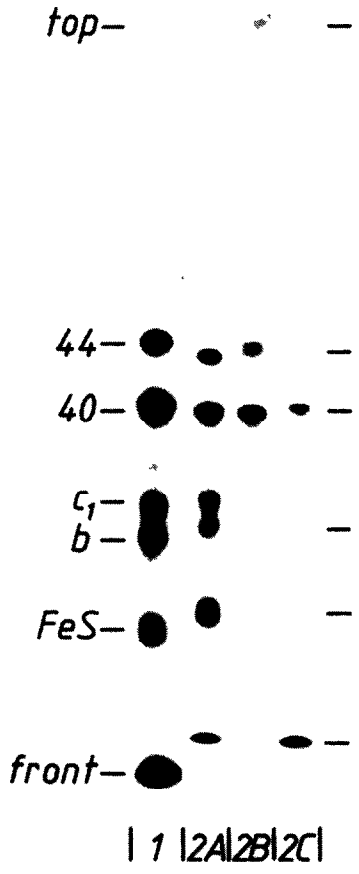

Fig.2. Efficiency of transfer. ^{125}I-Labelled cytochrome bc_1 complex was fractionated in two parallel lanes on an 11% SDS–polyacrylamide gel. After electrophoresis one lane was dried immediately; from the other, the protein was transferred to a double layer of nitrocellulose. Autoradiograms of the gel and filters are shown. Lane 1, original gel; 2A, first nitrocellulose filter after transfer; 2B, gel after transfer; 2C, second filter after transfer. Because of staining after the transfer, the gel has increased somewhat in size.

Table 1
Efficiency of protein transfer

Subunits	First filter	Second filter
44 000 M_r	65	–
40 000 M_r	70	10
Cytochromes b and c_1 (30 000 M_r)	65	12
FeS protein (25 000 M_r)	123	9
Small subunits (17 000, 14 000 and 11 000 M_r)	21	22

Autoradiograms presented in fig.2 were scanned at 550 nm. The values given are percentages of the amounts of protein originally present in the gel (fig.2, lane 1)

autoradiogram of a second filter which was placed on top of the first one in this experiment (lane 2C).

Scanning the autoradiograms presented in fig.2 shows that under the conditions used >65% of each individual subunit is retained on the filter (table 1). However, of the smallest subunits ($M_r < 17\,000$) a considerable amount leaks through the filter and is partially retained on the second filter. When the transfer is done for only 30 min at 40 V, the recovery of small subunits on the filter is almost complete (A. G. Jochemsen, personal communication).

We investigated whether these phenomena interfere with the quantitation of small amounts of protein in complex mixtures. Therefore, we have fractionated increasing amounts of yeast cell lysates, transferred them to a nitrocellulose filter and subsequently incubated the filter with a specific antiserum and ^{125}I-labelled protein A from *S. aureus*. The results are shown in fig.3A,B. It is clear that both the darkening of the autoradiogram and the amount of ^{125}I-labelled protein A present on the filter increase linearly with the total amount of protein applied to the gel, provided that this is ≤25 μg. Calculation shows that it is possible to detect 0.1 ng antigen after 16 h exposure of the autoradiogram (fig.3A, first lane).

This method is very well suited for precise characterization of a certain antiserum [6]. Moreover, the same filter can be used again for characterization of other antisera, as shown in fig.4. A replica of a gel on which a yeast-cell lysate was fractionated, was challenged with antiserum against the 44 000 M_r subunit of the cytochrome bc_1 complex (fig.4A). After autoradiography the immunodetection procedure was

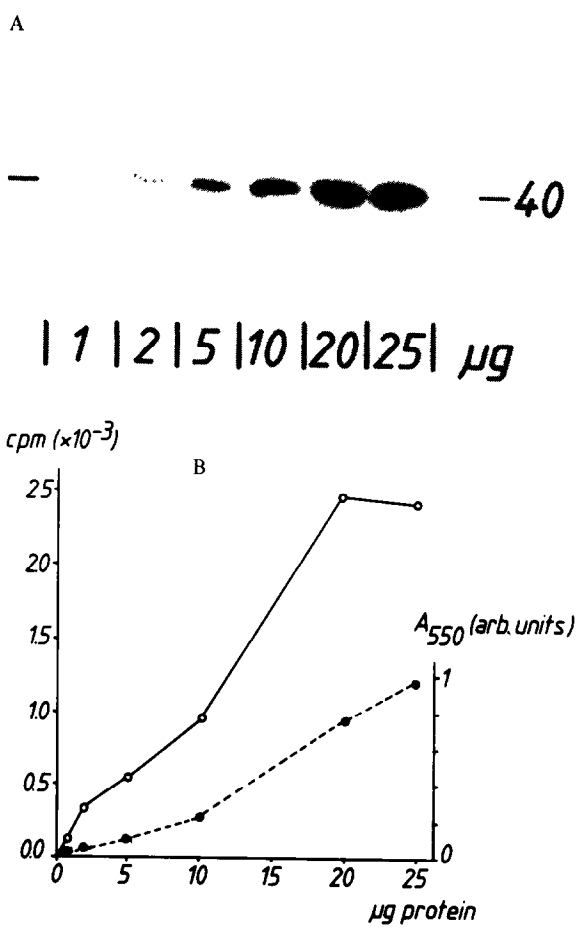

Fig.3. Quantitation of the immunodetection. Increasing amounts of cell lysate were fractionated on an 11% polyacrylamide gel and transferred to nitrocellulose. The filter was incubated with antiserum directed against the 40 000 M_r subunit of the cytochrome bc_1 complex and subsequently with ^{125}I-labelled protein A as described. The autoradiogram shown was exposed for 16 h (fig.3A). Similar autoradiograms were scanned at 550 nm (fig.3B, dotted line). The radioactive bands on the filter were cut out and counted as in section 2 (fig.3B, solid line).

repeated with a serum directed against the 40 000 M_r subunit of this complex (fig.4B) and finally with anti-hexokinase serum (fig.4C). Since the label bound to the filter during the first reaction is not washed away during the successive treatments, this leads to a composite pattern of bands.

In summary, we have shown that the procedure in [6] is a simple, rapid and sensitive technique to determine the concentrations of single proteins in complex mixtures, fractionated on SDS–polyacrylamide slab

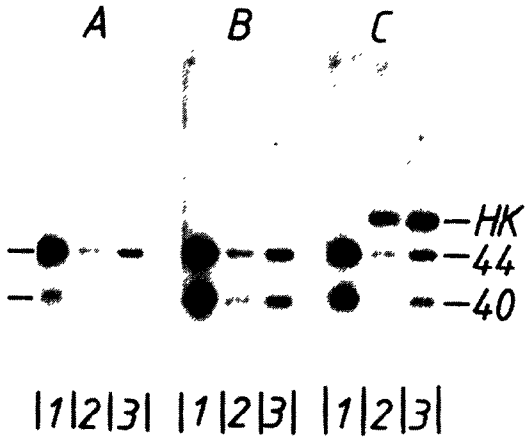

Fig.4. Sequential characterization of 3 different antisera using the same filter. On an 11% polyacrylamide gel, 5 μg cytochrome bc_1 complex (lane 1), 10 μg lysate of cells grown on 15% glucose (lane 2) and 10 μg lysate of cells grown on 2.5% lactate (lane 3) were fractionated. The proteins were transferred to a nitrocellulose filter, which was incubated with serum directed against the 44 000 M_r subunit, ^{125}I-labelled protein A and autoradiographed. The procedure was repeated with serum directed against the 40 000 M_r subunit (panel B) and with serum directed against hexokinase (HK) (panel C). The autoradiograms shown were exposed for equally long periods.

gels. The main advantage of the use of nitrocellulose filters over that of DBM-paper is that glycine and SDS, present in the gel, do not have to be removed prior to transfer [2,3] and that no activation of the filter is required. The whole procedure, including electrophoresis, transfer, immunodetection and autoradiography, usually takes only 48 h. This is considerably shorter than the immunoreplica technique [13] without loss of sensitivity [14].

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