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ANALYSIS OF STRUCTURAL CELL PROTEINS BY ISOELECTRIC FOCUSING IN ZWITTERIONIC AGAROSE GEL THIN LAYERS

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SUMMARY

A simple, rapid and reproducible analysis of structural cell proteins by isoelectric focusing in agarose thin layer gel is presented. The method consists of solubilization of total cellular proteins with a 5% zwitterionic detergent and isoelectric focusing in an 0.5-mm agarose gel containing 2% of the same detergent. A pH gradient formed by ampholytes was stabilized by using free amino acids in electrode solutions. The protein patterns of analyzed samples could be visualized by staining, direct autoradiography of ^{35}S or by fluorography of ^{3}H. The method was applied to analysis of measles virus proteins recovered by immunoprecipitation with mono- and polyclonal antibodies.

INTRODUCTION

Isoelectric focusing (IEF) is a high-resolution technique for the separation of proteins, based on the differences in their isoelectric points[1]. It has been also used for hydrophobic proteins solubilized by non-ionic detergents[2,3] or urea[4,5]. However, membrane proteins are dissolved incompletely under these conditions[2,3], and comparison of different samples with the same discriminative capacity as obtained by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)[6] has not been successful.

Since zwitterionic detergents possess both cationic and anionic groups, their binding to proteins does not alter the net charge of the macromolecule. Thus, these compounds, in contrast to ionic detergents (sodium dodecyl sulphate, deoxycholate, etc.) seem to be suitable for IEF[3,7]. Moreover, because of their charged groups, zwitterionic detergents solubilize structural cell proteins more effectively than do non-ionic detergents, but still preserve most of their enzyme activities[8]. In the present work a zwitterionic detergent was used for solubilization and fractionation of fibroblast membranes and measles virus proteins in agarose gel thin layers.

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MATERIALS AND METHODS

Cell cultures

Human synovial fibroblasts from synovial explants and VERO cells (African monkey kidney cells) were cultured on 57-cm^2 petri dishes (Nunc Products, Roskilde, Denmark) in Dulbecco's modification of Eagle's minimum essential medium (DMEM; Flow Laboratories, Irvine, U.K.) supplemented with 10% fetal calf serum (Flow) and antibiotics (100 I.U./ml penicillin G and 50 μg/ml streptomycin sulphate). Measles virus was grown in monolayer cultures of VERO cells infected with a wild-type measles virus strain[9].

Radiolabelling of the cells

Cellular proteins were labelled metabolically with [^{35}S]methionine in methionine-free medium for 18 h (200 μCi per 57 cm^2, over 1.000 Ci/mmol; The Radiochemical Centre, Amersham, U.K.). After labelling, the cells were scraped off with a rubber policeman into ice-cold isotonic sucrose solution and collected by centrifugation at 1000 *g* for 10 min. If not used immediately, the cell pellets were stored at −70°C.

The surface glycoproteins of synovial fibroblasts were labelled according to the galactose oxidase–NaB^3H_4 method of Gahmberg and Hakomori[10]. Radioactivity incorporated was measured by trichloroacetic acid (5%) precipitation after solubilization of the cells as described below.

Preparation and immunoprecipitation of cellular proteins for focusing

Cell pellets were lysed into 5% zwitterionic detergent (Zwittergent TM_{3-14}; Calbiochem-Behring, La Jolla, CA, U.S.A.) by a 2-min ultrasound treatment (Sonogen, Model D-50; Branson Europa N.V., Loosdrecht, The Netherlands). Cell lysates were centrifuged at 1.000 *g* for 10 min and aliquots of supernatants were used in IEF analysis or as starting material in immunoprecipitation after radioactivity determination.

Measles-infected and control VERO cells were lysed in 0.15 *M* phosphate-buffered saline (PBS), pH 7.4, supplemented with 5% zwitterionic detergent (zwitterion lysis buffer for IEF) or PBS supplemented with 1.0% Triton X-100, 0.5% deoxycholate (DOC) and 0.1% sodium dodecyl sulphate (SDS) (standard lysis buffer for SDS-PAGE). The lysates were clarified by centrifugation at 8900 *g* for 10 min. For the immunoprecipitation, 20 μl of antiserum were added to 30 μl of Protein-A Sepharose (Pharmacia Fine Chemicals, Sweden) in small conical tubes, which were continuously mixed at 37°C for 60 min, and the gel centrifuged. After two washings with PBS + 2% zwitterionic detergent or PBS + 0.5% Triton X-100, 40 μl of infected or control lysates (2 × 10^5 cpm each) were added. After incubation for 1 h at 37°C with constant mixing, the gel was washed twice in the corresponding washing buffer, once in distilled water with 0.1% zwitterionic detergent and finally suspended into small volumes of distilled water with 5% zwitterionic detergent. These were boiled for three min and the Sepharose removed by centrifugation. The supernatants were analyzed by IEF.

Isoelectric focusing in zwitterionic agarose gel thin layers

Analytical IEF was performed in horizontal slab gels in a Multiphor 2117

apparatus (LKB, Bromma, Sweden) as suggested by the manufacturer (Instruction No. 1881-A). Gels (26 × 12.5 × 0.05 cm) were formed between glass plates covered with GelBond® plastic sheets (LKB Producter No. 1850-101) as described by Jalkanen and Jalkanen[11]. The zwitterionic detergent (2%) was dissolved in water. The charge-balanced agarose (1%; LKB Producter No. 2206-111) was dissolved in zwitterionic solution on a bath of boiling water and cooled to 70°C. Prewarmed ampholytes (final concentration 3%; pH range of each IEF indicated in the figures) were mixed with agarose and injected with a prewarmed syringe between the plates along one side of the template frame, keeping the prewarmed mould vertical. The gel on the plastic sheet (on the hydrophilic side) was transferred to a humidity chamber and kept overnight at 10°C (zwitterionic detergent may precipitate and make the gels opalescent below 10°C).

When IEF was performed, the gel was placed on the top of the template lying on the cooling (+15°C) plate of the Multiphor, and strips soaked in the electrode solutions [anode, 0.05 *M* glycylglycine in 0.0043% phosphoric acid; cathode, 0.05 *M* L-lysine (free base) in 0.1% ethanolamine] according to Nicoletti *et al.*[12] were applied along the edges. After a 15-min prefocusing with 300 V, samples were applied on the gel using application pieces and focused for 30 min with 600 V. The application pieces were removed and the electrode strips replaced with new ones, which had been soaked in the same electrode solutions. After final focusing for 30–45 min with 1.000 V the gel was processed for protein staining and autoradiography, or fluorography.

Proteins were fixed in 10% trichloroacetic acid–1% sulphosalic acid for 5 min and washed twice in ethanol for 10 min. For fluorography the gels were then kept for 1 h in glacial acetic acid and 25% 2,5-diphenyloxazole (PPO). The PPO was precipitated into the gel by distilled water over 30–100 min[13]. The gel was dried with a stream of hot air and exposed to Kodak X-Omat film.

For protein staining, ethanol-washed gel was dried with a stream of hot air and stained with Coomassie Brilliant Blue R 250 (Gurr Products, Hopkin & Williams, Ronford, U.K.; 0.5 g per 100 ml destain solution) for 10–15 min. Finally the gel was twice destained for 10 min with the destain solution (38% ethanol and 10% acetic acid) and dried again. The ^{35}S- and ^{125}I-labelled proteins could be detected by direct autoradiography on Kodak X-Omat film.

RESULTS AND DISCUSSION

Recovery of structural proteins with zwitterionic detergent

Zwitterionic detergents have been shown to solubilize effectively hydrophobic proteins, like those of membranes[3,7]. In our hands non-ionic detergents, *e.g.* Triton X-100, and urea dissolved only half as much radioactivity from [^{35}S]methionine-labelled synovial fibroblasts as zwitterionic or ionic detergents (SDS, DOC) (data not shown).

We also studied immunoprecipitation of zwitterionic detergent-solubilized viral membrane proteins. Parallel cultures of measles virus-infected VERO cells were lysed both into zwitterion lysis buffer for IEF and standard lysis buffer for SDS–PAGE. The recoveries of radioactivity with different antisera revealed similar values (Table I), indicating equal solubilization of membrane proteins by both methods. The denaturation of proteins in both detergent solutions was insignificant because the antibodies precipitated the antigens from supernatants. However, low affinity anti-

TABLE I

IMMUNOPRECIPITATION OF POLYPEPTIDES FROM MEASLES VIRUS-INFECTED VERO CELLS LABELLED WITH [^{35}S]METHIONINE

A total of 200,000 cpm were precipitated with the indicated antisera. The results express the percentages of cpm precipitated (means of duplicates). Triton X-100 + DOC + SDS (TDS)– or zwitterionic detergent–lysis buffers were used in solubilization of cells as described in the text.

Precipitating antiserum	*Percentage of cpm precipitated*	
	TDS lysate	*Zwitterionic lysate*
Polyclonal rabbit antiserum to purified whole measles virus	7.6	5.0
Polyclonal rabbit antiserum to measles virus nucleocapsid proteins	4.6	2.4
Polyclonal rabbit antiserum to measles virus haemagglutinin*	1.7	1.6
Monoclonal mouse antiserum to measles virus haemagglutinin**	0.4	0.7

* For the description of polyclonal antisera see ref. 14.
** The description of monoclonal antiserum will be published elsewhere.

bodies, *e.g.* some monoclonal preparations, may need milder washing conditions than presented here, but then there is the possibility of unspecific background staining. Baileys *et al.*[8] have shown that membrane-bound enzymes also remain active in the zwitterionic detergent solutions.

Agarose thin-layer IEF in the presence of zwitterionic detergent

We started the present study by testing different detergent mixtures in the agarose gel for IEF. Urea added to agarose gel complicated the casting of the gel[4] and was avoided. With Triton X-100, proteins precipitated in the gel near the application site. This was overcome by the use of a zwitterionic detergent. Fig. 1 shows the distribution of [^{35}S]methionine-labelled polypeptides of synovial fibroblasts in two different pH gradients (lanes 1 and 2), and after surface labelling with the galactose oxidase–NaB^3H_4 method (lane 3). [^{35}S]methionine labelling revealed more than 30 protein bands in IEF in a broad pH gradient (lane 1). In a narrower gradient the resolution was not as sharp (lane 2). As expected, less bands were detected after surface labelling than after methionine labelling, but even the latter method produced about 20 different bands (lane 3). Lane 1 was obtained after partial overexposure, but still the amount of radioactive material at the application site was minimal.

We also analyzed immunoprecipitated measles virus proteins recovered from control and infected VERO cells (see Table I). To our surprise, both haemagglutinin specific antisera (poly- and monoclonal) produced a triplet protein pattern (Fig. 2: lanes 3 and 4), even though a single band has been observed in SDS-PAGE[14]. The triplet may represent three glycosylation forms of the main membrane protein of measles virus. Analysis of whole VERO cell lysates and polypeptides recovered with

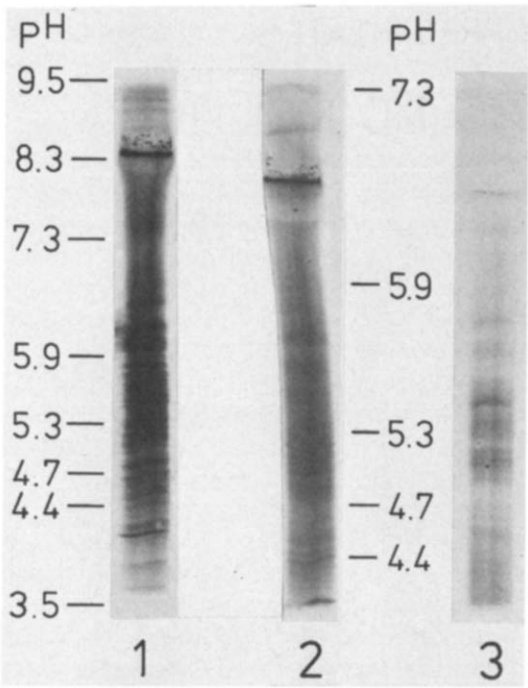

Fig. 1. Analysis of synovial fibroblast cellular proteins by isoelectric focusing in zwitterionic agarose thin-layer gel. Lanes 1 and 2 show polypeptides after metabolic labelling with [^{35}S]methionine and lane 3 after surface labelling with the galactose oxidase–NaB^3H_4 method. Lanes 1 and 2 were recovered by direct autoradiography and lane 3 by fluorography. The pH gradients (for lane 1 from 3.5 to 9.5 and for lanes 2 and 3 from 4.0 to 8.0) are indicated using the focused p*I*-standards (Serva protein test mixture 9 for IEF).

antisera against whole virus and virus nucleocapsid proteins (lanes 1 and 2) was visible from samples containing 500–2000 cpm each, after an exposing period of only 3 weeks on the X-ray film at −70°C. Thus the agarose gel thin layers also improved the sensitivity of the radioactivity detection. We have recently shown that proteins fractionated by IEF and transferred onto nitrocellulose sheets are immunoreactive and may be identified with different antisera and indirect immunoenzyme staining[11]. So this method is an alternative to the immunoprecipitation presented in the present paper.

Zwitterionic detergents are non-conductors and thus do not induce the heating effect which accompanies electrofocusing in the presence of ionic detergents[3,7], which may also alter the isoelectric points of proteins due to protein–detergent interaction[8]. Although the stability of the pH gradient is reported to be enhanced by zwitterionic detergents[7] (in contrast to ionic detergents), the stability was further improved with the help of free amino acids in the electrolytes, as described by Nicoletti *et al.*[12]. This was observed as a reduction of the waving of protein bands during IEF (not shown).

We find the presented IEF procedure to be the method of choice for IEF analysis of structural cell proteins, and to have wide applicability in biochemical research. It is also suitable for the first dimension of two-dimensional analysis of proteins and might increase the information obtained as the resolution of IEF is increased.

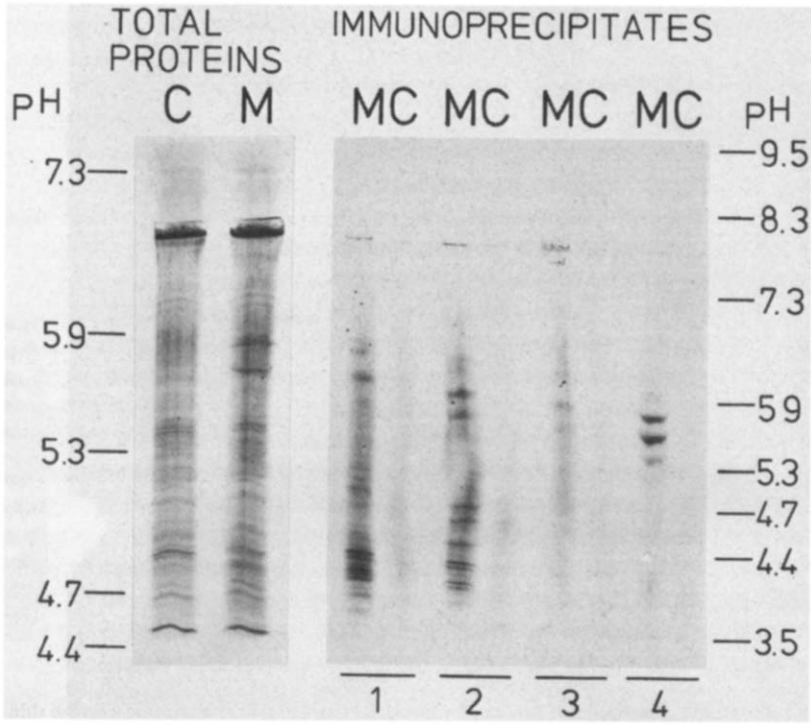

Fig. 2. Analysis of measles virus (MV) infected, [^{35}S]methionine-labelled cells by isoelectric focusing in zwitterionic agarose thin-layer gel. Total polypeptides of control (C) and infected (M) VERO cells without any immunoprecipitation are presented on the left. The isoelectric focusing pattern in lanes 1, 2 and 3 was produced from the material shown on the left but now immunoprecipitated with polyclonal rabbit antiserum to whole measles virus, to MV nucleocapsid proteins and to MV haemagglutinin, respectively, or with monoclonal mouse antibodies to MV haemagglutinin (lane 4). The pH gradients for both runs are indicated.

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