Journal of Chromatography, 527 (1990) 152–157
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5164

Note

Separation of phosphoproteins by fast protein liquid chromatography

PATRICIA LEONI[a,*], PAUL HEYWORTH and ANTHONY W. SEGAL

Department of Medicine, Faculty of Clinical Sciences, The Rayne Institute, University College London, London WC1E 6JJ (U.K.)

(First received April 7th, 1989; revised manuscript received November 29th, 1989)

Modification of proteins by phosphorylation is one of the major mechanisms by which intracellular events are regulated. A variety of cell functions including contraction, secretion, transport, biosynthesis and degradation of metabolites involve phosphorylation of proteins as a regulatory step [1–4].

The use of radioactively labelled phosphorus and electrophoresis in one dimension on polyacrylamide gels have been very useful tools to investigate protein phosphorylation [5–8], in particular when systems involved a relatively small number of proteins. A better resolution can be obtained using isoelectric focusing followed by electrophoresis on polyacrylamide gels, which allows identification of proteins in very complex mixtures without purification [9,10]. This method, however, is very laborious and has poor reproducibility, and even if reproducibility could be improved [11], only a very small amount of protein can be used in each run and proteins that are present in a small proportion in the mixture could be very difficult to visualise.

The use of fast protein liquid chromatography (FPLC) instead of isoelectric focusing allows the rapid separation of phosphoproteins from a complex mixture and can be easily manipulated to maximize information on the proteins of interest.

[a]Address for correspondence: Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, U.K.

0378-4347/90/$03.50

EXPERIMENTAL

Materials

Chemicals for electrophoresis were from Bio-Rad (Watford, U.K.), *n*-octylglucopyranoside was from Sigma (Poole, U.K.) and tissue culture products were from Gibco (Paisley, U.K.). All other chemicals were of analytical grade and were purchased from BDH (Poole, U.K.). Disposable plastic material was from Sterilin (Felham, U.K.). ^{32}P-Labelled phosphate was from Amersham International (Aylesbury, U.K.).

Isolation of phosphoproteins from primary cultures of fibroblasts

Human fibroblasts were isolated from connective tissue and cultured in RPMI 1640 medium containing 10% fetal calf serum. Subconfluent monolayers were washed with saline and labelled with $^{32}PO_4$ in a buffer containing 136 m*M* NaCl, 2.7 m*M* KCl, 1 m*M* $MgSO_4$, 1 m*M* $CaCl_2$, 5.6 m*M* glucose, 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 0.025% (w/v) bovine serum albumin and 0.2 mCi/ml $^{32}PO_4$, pH 7.4. After 1 h incubation, the cells were rapidly harvested by centrifugation and resuspended in a small volume of extraction buffer containing 50 m*M* Tris–HCl, 50 m*M* KCl, 50 m*M* NaF, 1% *n*-octylglucoside, 10 U/ml Trasylol, 0.5 m*M* phenylmethylsulfonylfluoride (PMSF) and 1 mg/l *p*-tosyl-L-lysine chloromethyl ketone (TLCK). The activity of the labelled extract is 2–3 μCi/μg of protein.

The suspension was sonicated with three 20-s bursts of amplitude 18 in a MSE Soniprep 150 and spun for 30 min at 73 000 *g* in a Sorvall OTD 50B ultracentrifuge.

Procedure for the separation of phosphoproteins

Anion-exchange chromatography was performed at room temperature on a Pharmacia FPLC system using a Mono Q HR 5/5 column (I.D. 5 mm, bed height 50 mm) at a flow-rate of 1 ml/min. The column was equilibrated with a buffer containing 50 m*M* Tris–HCl, 50 m*M* NaF, 0.8% *n*-octylglucoside, 0.5 m*M* PMSF, 10 U/ml Trasylol and 1 mg/l TLCK. The sample was injected into the column using a 2-ml loop. After washing the column with 5 ml equilibration buffer the proteins were eluted using a 50-ml linear gradient of NaCl in the same buffer. The eluted proteins were detected by their absorption at 280 nm. Buffers and extracts were filtered through a 0.22-μm membrane before use. Fractions of 1 ml were collected in Eppendorf tubes and immediately transferred to ice. Trichloroacetic acid (TCA) was added to each fraction to a concentration of 10% (w/v) and proteins were left to precipitate overnight. If the protein concentrations were very low, 5 μg of albumin was used as carrier.

Precipitates were washed with ethanol–diethyl ether (1:1) to eliminate TCA and neutralized with dilute NaOH if necessary. Further separation on a 12% polyacrylamide gel (30% T, 2.67% C) in the presence of sodium lauryl sulphate

(SDS-PAGE) was performed according to Laemmli [12] using a Bio-Rad Protean II slab cell. Gels were stained with Coomassie Blue R, dried under vacuum and autoradiographed with Hyperfilm HP (Amersham International). Isoelectric focusing was performed according to Giometti et al. [13].

RESULTS AND DISCUSSION

A very effective separation of phosphoproteins was obtained by the use of FPLC system. Whole cell protein extracts from human fibroblasts labelled in culture with ^{32}P were loaded on a Mono Q anion-exchange column and eluted with a linear NaCl gradient. *n*-Octylglucoside was included in all buffers to solubilize membrane proteins. This detergent has excellent solubilizing prop-

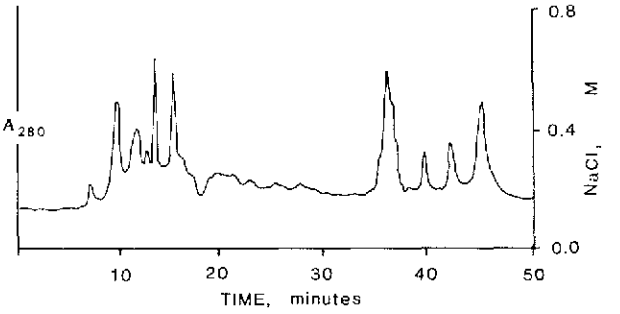

Fig. 1. Elution profile of protein extracts from human fibroblasts on a Mono Q HR S/S column, using a 0–0.8 *M* NaCl linear gradient. Detection was by absorption at 280 mm.

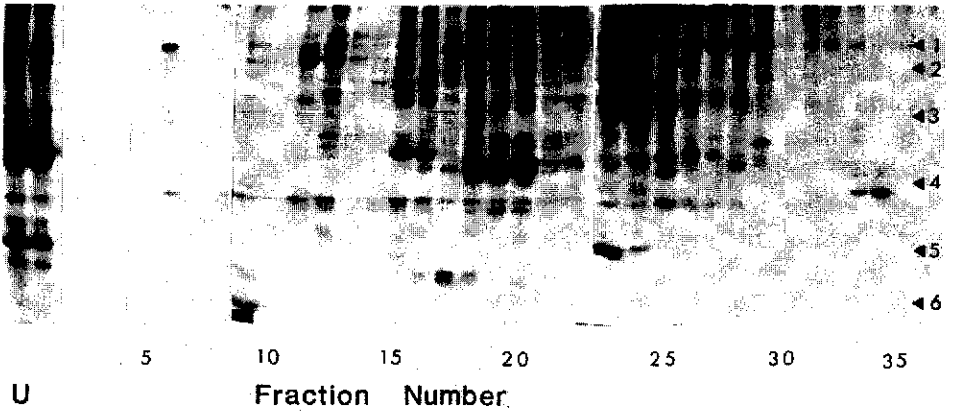

Fig. 2. Autoradiography of $^{32}PO_4$-labelled phosphoproteins analysed by SDS-PAGE. Fractions eluted from the Mono Q column were run on SDS-PAGE. After staining, the gels were dried and autoradiographed and the films were developed and photographed. Molecular mass standards: 1, 94 000; 2, 67 000; 3, 43 000; 4, 30 000; 5, 20 100; 6, 14 400.

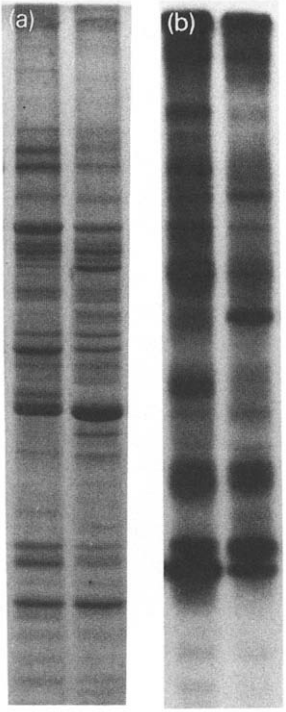

Fig. 3. Separation of $^{32}PO_4$-labelled phosphoproteins by SDS-PAGE and autoradiography. The sample consisted of fractions 12 and 13 from the separation on Mono Q of a $^{32}PO_4$-labelled extract of HeLa cells following the same protocol as for human fibroblasts. (a) Coomassie Blue staining; (b) autoradiography.

erties, does not absorb light at 280 nm and does not interfere with TCA precipitation of proteins. For cytosolic proteins the detergent is unnecessary. Up to 25 mg of protein can be loaded onto this column, but the process can be scaled up to 500 mg. The elution profile is shown in Fig. 1. Cationic proteins in the extract, 27% at pH 7.4, do not bind to the column. Separation of these proteins can be achieved on a Mono S cationic exchanger. Each of the fractions contains a mixture of proteins that is analysed on SDS-PAGE.

After electrophoresis, the phosphoproteins were localised by autoradiography (Fig. 2). Using a light box, 306 bands were clearly identified. A number of bands were visible after 2 h of film exposure, the majority appeared after one day. Even though the physicochemical interactions involved in ion-exchange chromatography have not been completely elucidated, the elution of proteins depends on the number and distribution of charges on the protein surface [14,15]. This dependence on charge makes the method suitable to replace isoelectric focusing as a first-dimension separation. When separating phosphoproteins, even if the resolution of the SDS-PAGE were very good, the bands or spots produced by $^{32}PO_4$ after autoradiography are not very sharp (Fig. 3). The background of the autoradiography darkens considerably with longer ex-

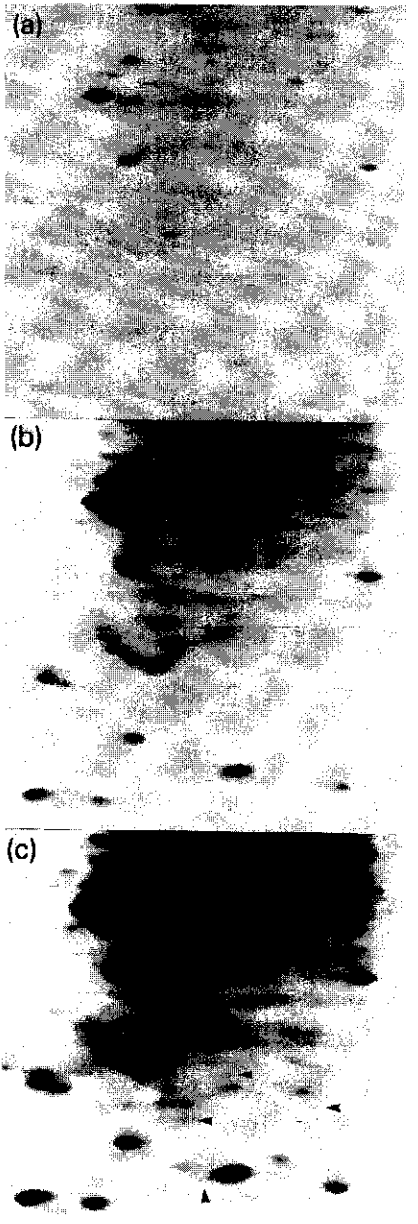

Fig. 4. Two-dimensional separation of $^{32}PO_4$-labelled phosphoproteins. Human fibroblast protein extracts were separated by isoelectric focusing followed by SDS-PAGE. The gel was dried and autoradiographed for different lengths of time: (A) 6 h; (B) 24 h; (C) 48 h.

posure times. Fig. 4C shows spots that only appear after 48 h exposure (arrows), after a $^{32}PO_4$-labelled cell extract has been analysed by isoelectric focusing followed by SDS-PAGE. Any light spot located near a very intense spot would be very difficult to see. Only a fraction of the whole gel is shown. It is therefore very important to separate the proteins as much as possible, and in this respect isoelectric focusing does not offer much flexibility. The detection of phosphorylation of a particular minor component may be of interest and the film might need several days of exposure. Because a Mono Q column can be loaded with milligrams of proteins rather than micrograms, and the fractions eluted are processed individually, the amount of protein loaded on each well in SDS-PAGE and the length of film exposure can be varied according to the extent of phosphorylation in each of them. Only a limited number of the fractions, generally from 6 to 38, contained phosphoproteins.

The chromatography is completed in 1 h, and once the proteins of interest are identified, the conditions of the run (shape of gradient, fraction volume and flow-rate) can be adjusted so that optimal separation of bands can be achieved and the separation time can be considerably reduced, as well as the number of fractions to be analysed by SDS-PAGE.

ACKNOWLEDGEMENTS

We thank the Wellcome Trust, Medical Research Council and the Special Trustees of University College Hospital for support.

REFERENCES

1 P. Cohen, Nature, 295 (1982) 613.
2 P. Cohen, Proc. R. Soc. London, B234 (1988) 155.
3 Y. Takai, V. Kikkawa, K. Kaibuchi and Y. Mishizuka, Research, 18 (1984) 119.
4 D.R. Sibley, J.L. Benovic, M.G. Caron and R.J. Lefkowitz, Cell, 48 (1987) 913.
5 J.C. Garrison, J. Biol. Chem., 253 (1978) 7091.
6 J.C. Garrison and J.D. Wagner, J. Biol. Chem., 257 (1982) 1313S.
7 A.W. Segal, P.G. Heyworth, S. Cockcroft and M.M. Barrowman, Nature, 316 (1985) 547.
8 P.G. Heyworth and A.W. Segal, Biochem. J., 239 (1986) 723.
9 L. Anderson and N.G. Anderson, Proc. Natl. Acad. Sci. U.S.A., 74 (1977) 5421.
10 L.E. King, Jr., P. Carpenter and S. Cohen, Biochemistry, 19 (1980) 1524.
11 S.M. Hanash and J.R. Strahler, Nature, 337 (1989) 485.
12 U.K. Laemmli, Nature, 227 (1970) 680.
13 C.S. Giometti, N.G. Anderson and N.L. Anderson, Clin. Chem., 25 (1979) 1877.
14 M.T.W. Hearn, A.N. Hodder and M.I. Aguilar, J. Chromatogr., 458 (1988) 27.
15 W.R. Melander, Z. El Rassi and C. Horvath, J. Chromatogr., 469 (1989) 3.