Analysis of mitogenic activity of proteins after separation by gel electrophoresis

Otmar Hohenwarter, Gorji Marzban, Elisabeth Jisa, Karola Vorauer-Uhl & Hermann Katinger
Institute of Applied Microbiology, University of Agricultural Sciences, Vienna, Austria

Received 1 April 1998; accepted 26 September 1998

Key words: gel electrophoresis, growth factor, *in vitro* assay

Abstract

We have used a combination of gel electrophoresis and a cell culture assay in microplates to analyse mitogenic activity in tissue extracts. The procedure is a modification of the method described by Kuo et al.. The proteins were separated by native gel electrophoresis or isoelectric focusing. The gel was sliced and defined pieces were transferred into tissue culture inserts fitting in 96 well microplates, which contained the test cells. The proteins diffused from the gel slices directly into the culture supernatant and the mitogenic effects were evaluated by a colorimetric assay (MTT or phosphatase activity). Human interleukin 2 was used to demonstrate the feasibility of the method by evaluating the mitogenic effect on the cell line CTLL-2. Extracts of bovine pituitary glands were separated by native gel electrophoresis and isoelectric focusing and several protein bands could be identified which showed a distinct mitogenic effect on human endothelial cells. The method is very sensitive and allows rapid screening of protein mixtures for bioactive fractions.

Abbreviations: rh-IL2 – recombinant human interleukin 2; SOD – superoxiddismutase; ECGS – endothelial cell growth supplement; MTT – 3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazoliumbromid; PAGE – polyacrylamid gel electrophoresis

Introduction

Cultivation media for animal cells frequently contain complex additives like tissue extracts or sera which induce cell division. In many cases defined growth factors could be isolated from such additives and purified proteins are available instead of undefined mixtures. Nevertheless complex additives are still in use and polypeptides which regulate cell growth remain to be isolated.

Complex mixtures of proteins may be separated rapidly by *Phast* system gel electrophoresis and tested subsequently in cell culture. Using transwell inserts in microwell plates, gel slices were eluted directly into the cell culture supernatant and the mitogenic effect was evaluated by H^3 -thymidine incorporation (Kuo et al., 1991). We used a modification of this procedure in conventional slab gels to be able to apply sample volumes up to 0.1 ml. In order to avoid the use of

radioactivity enzymatic assays for the mitogenic effect were applied.

Two examples were used to evaluate the feasibility of the method:

- a purified recombinant fusion protein of human superoxid dismutase and interleukin 2 (SOD-IL2)
- an extract from bovine pituitaries

The recombinant fusion protein SOD-IL2 was produced in CHO cells. The purified protein shows SOD and IL2 activity (Vorauer-Uhl, 1993). For determination of the biological activity of IL2 a cell culture assay has been described (Gillis et al., 1978). It is based on the cell line CTLL-2 which is strictly dependent on IL2 in the culture medium.

Extracts from bovine pituitary glands are used as cell culture additive for epithelial and endothelial cells and are known to contain several polypeptide growth factors like members of the FGF family. We used hu-

Figure 1. Diagram of the test procedure: After electrophoresis gel slices were transferred into microwell inserts in a 96 well plate in which test cells were grown. Separated proteins diffused directly into the culture medium and active fractions stimulated a mitogenic response.

man umbilical vein endothelial cells to evaluate the mitogenic response.

Materials and methods

Cell culture

Cell culture medium and foetal calf serum were obtained from Biochrom (Berlin, Germany). Cell culture reagents were purchased from Sigma (St. Louis, USA), plastic ware from Nunc (Roskilde, Denmark).

Routine cell culture was performed in Roux bottles in a humidified atmosphere containing 6% CO₂.

Preparation and cultivation of human umbilical vein endothelial cells has been described elsewhere (Hohenwarter et al., 1992). The culture medium for endothelial cells was M199 plus 15% foetal calf serum, 100 µg ml⁻¹ endothelial cell growth supplement (ECGS) and 90 µg ml⁻¹ heparin. ECGS is an extract of bovine brain and was prepared in our laboratory according to the method of Maciag et al. (1979). CTLL-2 cells (ATCC No TIB 214) were cultivated in suspension culture in medium RPMI 1640 plus 10% foetal calf serum, 110 mg l⁻¹ sodiumpyruvate and 20 U ml⁻¹ rh-IL2 (Boehringer Mannheim, Germany). The splitting ratio was 1:100 twice a week.

The cell lines were routinely checked for mycoplasma contamination using the Hoechst stain method.

Extraction of pituitary glands

Bovine pituitary glands were removed from 4 to 9 year old healthy cows in the slaughterhouse and frozen

immediately in liquid nitrogen. Frozen tissue was pulverised and 1 g was dissolved in 15 ml 0.1 M (NH₄)₂SO₄ solution (pH 4) by stirring for 1 h at 4 °C. Insoluble material was separated by centrifugation (30 min, 16,000 × g). The protein content of the extract was 30 mg ml⁻¹ as determined by the BioRad protein assay.

Separation and bioassay

Isoelectric focusing:

Polyacrylamide (National Diagnostic) gels (T 5%, C 3%) were prepared and washed at least 6 times to remove acrylic acid residues prior to drying. The gels were reswollen in a solution containing 2.4 ml pharmalytes and 1.2 g glycerol (Merck, Germany). Distilled water was added to a volume of 30 ml and mixed. The gels were rehydrated for at least 1 h before using. The pH gradient was to be formed by a 30 min pre-focusing in a *Multiphor II* system (Pharmacia, Sweden). The samples were focused afterwards for 2 h at 2000 V and 15 mA. For the pI range 5–8 as cathodic solution 1 M NaOH (Merck, Germany) and as anodic solution 40 mM glutamic acid (Sigma, USA) were used. For pI range 3–10 we used as cathodic solution 1 M NaOH (Merck, Germany) and as anodic solution 40 mM aspartic acid (Sigma, USA).

Native electrophoresis:

Pre-cast polyacrylamide gels (*Clean gel* for native PAGE from Pharmacia, T 5% stacking gel and T 10% separation gel) were used. All gels had to be washed at least 6 times for 30 min with distilled water in order to remove NaN₃ residues. The buffer systems were

Figure 2. Isoelectric focusing of SOD-IL2: Purified SOD-IL2 fusion protein (100 ng) was focused (pI 3-10) and the mitogenic activity of gel slices (indicated by numbers) on CTLL-2 cells was determined by a MTT test. A parallel lane shown below was stained and several isoforms of the proteins were detected, which were all active in the bioassay. An empty gel slice was used as negative control and 3 ng/ml SOD-IL2 was the positive control.

prepared according to the manufacturer's instruction but without cell toxic conservation agents.

After separation one lane was cut in equal pieces (2×5 mm). The slices were transferred into tissue culture inserts (8 well strip inserts from Nunc, pore size $0.2 \mu\text{m}$) which contained $40 \mu\text{l}$ culture medium (Figure 1). The inserts were placed in 96-well microplates which contained the test cells (CTLL-2: 3,000 cells per well, endothelium: 1,500 cells per well). For the bioassays antibiotics (100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, 250 U ml^{-1} nystatin) were added to the culture medium, IL2 or ECGS were omitted. In parallel wells 3 ng ml^{-1} SOD-IL2 or $200 \mu\text{g ml}^{-1}$ ECGS plus $90 \mu\text{g ml}^{-1}$ heparin were added as positive control. After 24 h the inserts were removed. After 72 h the mitogenic activity on CTLL-2 cells was evaluated by a MTT test procedure (details described by Hohenwarter et al., 1996). The effect on human endothelial cells was evaluated after 96 h using the acid phosphatase assay (Connolly et al., 1986). Be-

fore the enzymatic assays (MTT or acid phosphatase assay) were started, the wells were examined under the microscope to estimate by eye which gel slices induced cell growth. The results which were obtained by microscopic observation correlated very well with the results of the enzymatic assays.

Parallel lanes of the gel were stained by silver staining (Rabilloud et al., 1988).

Results

Isoelectric focusing of SOD-IL2

Purified SOD-IL2 (100 ng) with a known isoelectric point of 5.85 (Vorauer-Uhl, 1993) was focused on gels in a pI range 3-10. The protein has several isoforms which were visualised by silver staining. A parallel lane was sliced and the fractions were tested in the microwell inserts for growth stimulating effects on CTLL-2 cells. After 4 days examination under the mi-

Figure 3. Isoelectric focusing of pituitary extract: 50 μ l bovine pituitary extract (containing 1.5 mg protein) were separated by isoelectric focusing (pI 5–8). Gel slices indicated by numbers were tested in the microwell assay with endothelial cells. The mitogenic activity was evaluated by a phosphatase assay. The most active fraction was found between pI 5.3 and 6.2. ECGS was used as positive control.

croscopie revealed substantial cell growth in the wells which contained slices 2 to 7. In the other wells the cells started disintegration, since they cannot survive without IL2 activity in the culture medium. The same slices were active in the MTT assay, slices 3 to 7 contained visible protein bands (Figure 2). Slice 2 was cut from a region where no protein was detected by staining. Nevertheless mitogenic activity was found.

Isoelectric focusing of pituitary extract

Fifty μ l pituitary extract were separated by isoelectric focusing (pI 5–8). A bioassay of the gel slices was performed with human endothelial cells. A broad region with mitogenic activity was found (Figure 3). The most active fractions ranged between pI 5.3 and 6.2. The maximum growth promoting activity was comparable to the endothelial cell growth supplement (ECGS) which is routinely used as rough growth factor preparation for the cultivation of endothelial cells.

By silver staining a protein smear rather than single bands were observed because of the high protein amount (1.5 mg) which was applied to the gel.

Native gel electrophoresis of pituitary extract

Next native gel electrophoresis of pituitary extract was performed. 20 μ l of pituitary extract were separated either under acidic (pH 5.5) or basic (pH 8.9) conditions. Gel slices were again tested for mitogenic activity on human endothelial cells. In acidic gels mitogenic fractions could be identified (Figure 4). The active region was sharper than the region obtained by isoelectric focusing. The mitogenic activity was about 75% of the positive control (ECGS). Similar results were obtained after electrophoresis under basic conditions (data not shown).

Discussion

The bioassay described in this study has several advantages. It is a useful combination of rapid separation by electrophoresis and evaluation of cell growth in microwell plates. It avoids the use of radioactivity and only small amounts of material are needed. Since most growth factors are active in very low concentrations,

Figure 4. Native electrophoresis of pituitary extract: 20 μ l of pituitary extract (containing 0.6 mg protein) were separated by native gel electrophoresis under acidic conditions (pH 5.5). The mitogenic activity of the gel slices on endothelial cells was determined by a phosphatase assay. ECGS was used as positive control.

the cell culture assay allows the detection of proteins in the gel which cannot be visualised by the most sensitive staining techniques. We have shown that the recombinant fusion protein SOD-IL2 remains biologically active during the isoelectric focusing procedure and diffusion from the gel into the culture medium. When we used the fusion protein SOD-IL2 for evaluation we found mitogenic activity in a region on the top of the gel where no protein bands could be detected by the silver staining method. Since 3 ng/ml SOD-IL2 are sufficient to promote rapid cell growth of CTLL-2 cells we were not surprised to detect mitogenic activity below the sensitivity level of the staining method.

We have chosen a 24 h period for elution of the proteins from the gel slices into the culture medium. Kuo et al (1991) could show that 95% of human epidermal growth factor diffused from *Phast* gels in the medium within 1 h. Since we used conventional slab gels we

have prolonged the elution time. No difference could be observed between a elution period of 24 h and 4 d.

The increase of the cell number in positive micro-wells is easily observed under the microscope. Since we never found any discrepancy between microscopic examination and the results of the enzymatic assay, we are sure that the results reflect changes in cell number. No unspecific effect of the pituitary extract on the enzymatic reaction itself has been observed.

The use of transwell inserts is essential to separate the gel slices from the cells to avoid inhibitory effects which would appear when the gel pieces were placed directly on the cell monolayer. Toxic effects of the gel which were observed in preliminary experiments could be minimised by extensive washing of the gels before electrophoresis.

The growth promoting activity of bovine pituitary extract on endothelial cells shows a broad peak after

isoelectric focusing. This could be due to different proteins or different isoforms of one protein. The pituitary is a source of many mitogenic peptides for example FGFs, EGF, TGFs and IGFs (Houben and Deneef, 1994) and probably several unknown ones. The separation in narrow pI ranges will allow a more detailed characterisation of the mitogenic proteins. The combination of gel electrophoresis and a cell culture assay in microwell plates has proven to be a rapid and sensitive method to screen for mitogenic fractions.

References

- Connolly DT, Knight MB, Harakas NK, Wittwer AJ and Feder J (1986) Determination of the number of endothelial cells in culture using an acid phosphatase assay. *Anal Biochem* 152: 136–140.
- Gillis S, Ferm MM, Ou W and Smith KA (1978) T cell growth factor: parameters for production and a quantitative microassay for activity. *J Immunol* 120: 2027–2032.
- Hohenwarter O, Schmatz C and Katinger H (1992) Stability of Von Willebrand Factor production in different human endothelial hybrid cell lines. *Cytotechnol* 8: 31–37.
- Hohenwarter O, Waltenberger A and Katinger H (1996) An *in vitro* test system for thyroid hormone action. *Anal Biochem* 234: 56–59.
- Houben H and Deneef C (1994) Bioactive peptides in anterior pituitary cells. *Peptides* 15: 547–582.
- Kuo K, Yeh H, Chu DZJ and Yeh Y (1991) Separation and microanalysis of growth factors by Phast system gel electrophoresis and by DNA synthesis in cell culture. *J Chromatography* 543: 463–470.
- Maciag T, Cerundolo J, Ilsley S, Kelley PR and Forand R (1979) An endothelial cell growth factor from bovine hypothalamus: Identification and partial characterisation. *Proc Nat Acad Sci USA* 76: 5674–5678.
- Rabilloud T, Carpentier G and Tarroux P (1988) Improvement and simplification of low-background silver staining of proteins by using sodium dithionite. *Electrophoresis* 9: 288–291.
- Vorauer-Uhl K (1993) Expression, Reinigung und Charakterisierung eines SOD-IL2-Fusionsproteins. Thesis, University of Agricultural Sciences, Vienna.
- Address for correspondence:* Otmar Hohenwarter, Institute of Applied Microbiology, University of Agricultural Sciences, Muthgasse 18, A-1190 Vienna, Austria.
E-mail: hobl@mail.boku.ac.at