Isoelectric focusing–polynucleotide/polyacrylamide-gel electrophoresis

A technique to separate and characterize nuclease activities

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Individual native nuclease activities from human leucocytes are separated by using two-dimensional gel electrophoresis in an apparatus that allows the simultaneous running of 28 gels. Proteins are separated by isoelectric focusing in a disc gel, followed by electrophoresis into a slab gel containing DNA. Protein denaturants are avoided in the second dimension by the use of a running pH well above the optimal pH for DNAase (deoxyribonuclease) activity. Electrophoresed gels are incubated in appropriate buffers to activate nuclease activity. After staining for intact DNA, the positions of active enzymes, unobscured by the presence of other proteins, are revealed as colourless spots in a reddish-purple field. The technique is easy to use and is sensitive to 50 pg of DNAase I. Versatility is provided by the use of either acidic or basic electrophoresis running buffers and by the use of specific gel incubation conditions to reveal different sets of enzyme activities. Two DNAases active at pH 7.4 in the presence of Mg^{2+} and Ca^{2+} , and sixteen DNAases active at acidic pH and not requiring metals, are detected. Treatment of the human enzymes with specific glycosidases reveals that many of the human DNAases are glycoproteins containing negatively charged moieties and may be derived from modification of parent activities.

Nuclease activities have been detected in a wide variety of cells and physiological fluids (Levy & Karpetsky, 1981). In most cases a spectrophotometric procedure was used to quantify the enzyme-catalysed degradation of a high- M_r polynucleotide (Karpetsky *et al.*, 1977). Unfortunately, such measurements do not yield information on the numbers and characteristics of isoenzymes unless activities are separated before assay. Although protein purification techniques, such as different types of column chromatography, can be used to study multiple nucleases (Naskalski, 1972;

Yamanaka *et al.*, 1977), such methods are time-consuming and do not lend themselves easily to examination of many samples.

These difficulties can be overcome by the use of polyacrylamide-gel electrophoresis to separate, identify and characterize multiple nuclease activities from multiple samples simultaneously. Separation of proteins by mass and charge is done easily by using any of a variety of one-dimensional electrophoresis systems (Gaal *et al.*, 1980). Nucleases can then be localized by several methods, including assaying for activity in individual gel slices (Zollner *et al.*, 1974a) or diffusion of low- M_r nucleic acid into an intact gel with subsequent incubation and staining of substrate to reveal enzyme positions (Wilson, 1969, 1971). A superior technique involves the inclusion of high- M_r substrate within the gel. This procedure reduces the number of steps required to detect activity and requires only that the enzymes be inactive and that the substrates be immobile during electrophoresis (van Loon, 1975). However, separations achieved with this method generally have been insufficient

Abbreviations used: %T denotes the total percentage concentration of both acrylamide (*a*) and *NN'*-methylenebisacrylamide (*b*) and is calculated by using the following formula: $(a+b) \times 100/v$, where *a* and *b* are in grams and *v*, the total volume, is in ml; %C is the percentage concentration of the cross-linker relative to the total concentration: $b(100)/(a+b)$; when *NN'*-diallyltartardiamide (DATD) is substituted for *NN'*-methylenebisacrylamide, the following notation is used: %C (DATD); endo-H, endo- β -*N*-acetylglucosaminidase, SDS, sodium dodecyl sulphate.

for detailed characterization and analysis of individual enzyme activities (Zöllner *et al.*, 1974a,b, 1978).

Improved resolution is obtained by adaptation of two-dimensional electrophoresis technology in which native proteins first are sorted by isoelectric points in a disc gel, with further resolution of native proteins in a slab gel containing polynucleotide, and subsequent detection of nuclease activities *in situ*. Furthermore, the development of an apparatus that provides for running 1–28 samples, coupled with the choice of either acidic or basic running systems, make this technology as versatile and simple as disc electrophoresis.

Materials and methods

Materials

Materials were obtained from the following sources: carrier ampholytes (LKB Instruments, Rockville, MD, U.S.A.); bovine pancreas DNAase I [Worthington (DPFF grade)]; Calbiochem, La Jolla, CA, U.S.A.; Miles Laboratories, Elkhart, IN, U.S.A.; Sigma, St. Louis, MO, U.S.A. (type I); Boehringer-Mannheim, Indianapolis, IN, U.S.A. (grade I); nylon hardware for two-dimensional electrophoresis apparatus (Small Parts, Miami, FL, U.S.A.); proteinase-free endo-H from *Streptomyces plicatus* (Health Research, Albany, NY, U.S.A.); neuraminidase from *Vibrio cholerae* and *Arthobacter ureafaciens* (Calbiochem-Behring, La Jolla, CA, U.S.A.) and from *Clostridium perfringens* (Worthington). Other materials were obtained from the sources given previously (Brown *et al.*, 1982).

Enzyme assays

Neuraminidase was assayed by the method of Cassidy *et al.* (1965), as modified in the Worthington (1982) catalogue. Endo-H was assayed by Health Research by using the procedure of Tarentino (1978). α - and β -mannosidase and β -galactosidase were not detected on incubation of endo-H (5 units/ml) for 21 h at 37°C with the respective *p*-nitrophenyl glycosides. Similar treatment revealed the presence of 300 μ -units of β -*N*-acetylhexosaminidase/ml.

Sample preparation

Cell samples were prepared and electrophoresis and detection of enzymes performed as outlined in Fig. 1. Cells were isolated and cell-free supernatants prepared as described previously (Brown *et al.*, 1982). Just before isoelectric focusing, supernatant equivalent to the contents of 10^6 cells was diluted to 14 μ l with sample buffer and 1 μ l of carrier ampholyte mix (1:1.5:7 mixture of pH 5–7, pH 9–11 and pH 3.5–11 carrier ampholytes) was

added. Where specified, supernatant from 4×10^7 sonicated cells was incubated with each glycosidase in 0.1 M-sodium acetate buffer, pH 5.5, to a final volume of 0.2 ml at 25°C. Portions were removed at various times and stored at –20°C. Reaction mixtures not containing glycosidases served as control samples.

Isoelectric focusing

Isoelectric-focusing gels (2 mm $\times$ 95 mm), prepared basically as described by O'Farrell (1975), contained 6.8% (w/v) acrylamide, 1.8% (w/v) *NN'*-methylenebisacrylamide/0.035% *NNN'N'*-tetramethylethylenediamine, 0.05% ammonium persulphate, 22.5% (v/v) glycerol and 2% ampholyte mix. Gels were prefocused at 4°C using 200 V (15 min), 300 V (30 min) and 400 V (30 min). Samples were focused at 400 V for 17–20 h (4°C). pH profiles of the focused gels were determined on at least one gel per run that was electrophoresed without a protein sample. pH was measured on the gel at 4°C with a LKB Multiphor microelectrode (2117-1111) and a Radiometer pH-meter (model PHM 64).

Polynucleotide/polyacrylamide-gel electrophoresis

Migration towards the anode (basic system). Isoelectric-focused disc gels were placed in the 1 mm $\times$ 0.5 mm grooves of the glass plates (Fig. 2a, 1). Then the plates, separated by 1 mm plastic spacers, were carefully stacked in the gel holder. A Plexiglass plate (Fig. 2a, 2) containing three holes was placed in the holder (Fig. 2a, 3) above the glass plates, and the screws tightened until the plates were held firmly in place. The assembled gel holder was tilted and oriented in the slab-casting apparatus (Fig. 2a, 4) such that the sides of the plates containing disc gels rested against the back of the apparatus. The tilt of the carrier permitted air bubbles trapped between plates to escape when the slab gels were poured. The slab gel solution [6.8% (w/v) acrylamide/0.18% (w/v) *NN'*-methylenebisacrylamide/0.02% (w/v) DNA/ 0.315 M-Tris/HCl (pH 8.9)/0.035% *NNN'N'*-tetramethylethylenediamine/0.08% ammonium persulphate] was poured into the slab-casting apparatus. Bubbles trapped between plates were released by tapping the apparatus. A wedge was added to conserve polynucleotide-containing solutions and to prevent formation of a large block of polyacrylamide along one side of the plate holder (Fig. 2a, 5).

After polymerization, excess acrylamide was removed with a razor blade. The sides and bottom of the gel holder (Fig. 2b, 2) were covered with a thin layer of high-vacuum silicone grease (Dow Corning Corp., Midland, MI, U.S.A.). The holder then was placed in the electrophoresis apparatus (Fig. 2b, 1) in contact with two rubber gaskets,

Fig. 1. Isoelectric focusing–polynucleotide/polyacrylamide-gel electrophoresis flow chart

Fig. 2. *Isoelectric focusing-polynucleotide/polyacrylamide-gel electrophoresis apparatus*

Nylon hardware and Plexiglass were used except as noted. (a) Gel casting equipment: 1, individual glass plate grooved to hold two disc gels after isoelectric focusing and having 1 mm-thick plastic spacers; 2, cover for top glass plate; 3, holder for up to 14 plates; 4, gel-casting chamber; 5, wedge used to conserve casting solution. (b) Electrophoresis equipment: 1, electrophoresis chamber having groove and two gaskets in centre to receive the gel holder (26-gauge platinum wire runs from the posts at one end of the apparatus to the sides of the buffer chambers); 2, gel holder containing DNA slab gels cast on the sides of isoelectric-focused disc gels; 3, top of electrophoresis chamber having ports for the addition of running buffer.

which were cut from $\frac{1}{8}$ in (~ 3.2 mm) Aimont sponge rubber (CS42; Phelps Rubber and Packing Co., Baltimore, MD, U.S.A.). The gaskets were held in grooves in the apparatus with silicone glue and seal (General Electric Co., Waterford, NY, U.S.A.). The gel holder was held tightly in place by the thumb screws on the top of the apparatus (Fig. 2b, 3). The chambers were filled with equal volumes of running buffer, and Bromophenol Blue tracking dye to a final concentration of 0.01% was added to the catholyte. Gels were electrophoresed at 300 mA per 28 gels (4°C) until the tracking dye traversed the width of the slab gel. After removal of the gels, one corner was removed to indicate the acidic end of the focused gel.

Migration towards the cathode (acidic system). The procedure was similar to that outlined above

except that the slab-gel solution contained 6% (w/v) acrylamide, 0.26% (w/v) *NN'*-methylenebisacrylamide, 0.02% DNA, 0.044 M β -alanine, 17.5 mM-acetic acid, 0.46% *NNN'*-tetramethylethylenediamine and 0.034% ammonium persulphate. The running buffer contained 0.35 M β -alanine and 0.14 M-acetic acid. Spermine hydrochloride (50 mM) and Methylene Green (0.01%) were added to the anolyte.

Detection of enzyme

Individual gels were incubated overnight in incubation buffer [either (i) 50 mM-citrate/phosphate buffer, pH 4.0, or (ii) 100 mM-Tris/HCl buffer, pH 7.4, containing 2.5 mM-MnCl₂ and 0.1 mM-CaCl₂, or (iii) 100 mM sodium acetate buffer, pH 4.6] in a shaker at 37°C . DNA in the

gels was stained with 0.1% Pyronin-Y in 7% (v/v) acetic acid for 6 h at 37°C. Gels were destained in 7% acetic acid.

Proteins in the gels were fixed and stained with Coomassie Blue as recommended by Pharmacia (Piscataway, NJ, U.S.A.).

Results and discussion

Isoelectric focusing and polynucleotide/polyacrylamide-gel electrophoresis

The establishment of pI equilibria was demonstrated by the less than 0.1 pH unit difference being found among the pI values measured on gels having 7.0%T/2.6%C or 4.0%T/5.4%C or gels of much larger pore size, such as those described by Baumann & Chrambach (1976) that contained 5.0%T/15%C (DATD). Furthermore, identical DNAase patterns were obtained from application of the sample anodically and cathodically. Presumably, rapid equilibria result from the low M_r of the enzymes examined. This contention is supported by the fact that DNAases from a variety of mammalian sources have M_r values of less than 38000 (Liao, 1981) and the six major human leucocyte DNAases have M_r values of less than 50000 (Brown *et al.*, 1982; Karpetsky *et al.*, 1982).

To facilitate isoelectric focusing-polynucleotide/polyacrylamide-gel electrophoresis, the apparatus depicted in Fig. 2 was designed. Up to 28 slab gels are polymerized in a gel carrier, which then is used directly in the electrophoresis apparatus, minimizing gel handling. Excellent reproducibility is demonstrated by the obtainment of 28 identical enzyme patterns after running 28 replicates. Furthermore, virtually identical enzyme patterns are obtained from the same sample electrophoresed at intervals over a period of months. Because the slab gels are narrow (25 mm), running time is rapid (~1.5 h at 300 mA/28 gels). Problems do not arise from Joule heating, owing to the large volumes (750 ml/chamber) of cooled buffer in intimate contact with the ends of the gels, glass plates and carrier during electrophoresis. Also, because both the top and bottom of each slab gel are covered by glass plates, there are no gradients of gas or hydration through the gels which could give rise to variations in field strength (Chrambach, 1980).

Although the slab gels used in isoelectric focusing-polynucleotide/polyacrylamide-gel electrophoresis occupy only one-sixth the area of conventional slabs, resolution of nuclease activities is very good (Fig. 3). This high-quality resolution results from detecting only a subset of all the proteins present, and from the efficient sorting of enzymes by isoelectric point in a long gel (9.7 cm), followed by sorting on the basis of additional criteria in the

Fig. 3. Human intracellular DNAase activity patterns obtained by isoelectric focusing-polynucleotide/polyacrylamide-gel electrophoresis

Samples were prepared and gels run as described in the Materials and methods section. (a) 10^5 total cells from a patient having acute progranulocytic leukaemia before treatment. (b) 5×10^5 mononuclear cells from an untreated acute-myelogenous-leukaemia patient.

second dimension. Since much longer DNA-containing slab gels (120 mm) do not show additional activities, the narrow slabs are suitable for our purposes.

The following evidence indicates that isoelectric focusing-polynucleotide/polyacrylamide-gel electrophoresis is a true two-dimensional technique. (1) Proteins and DNAase activities are distributed throughout the second dimension, with some DNAases having different R_m values but virtually identical pI values. (2) Nucleases requiring alkaline pH for optimum activity are resolved from the acidic enzymes [compare Figs. 4(b) and 4(d), particularly at pI 5.5, where relative mobilities in the second dimension are quite distinct for the acidic enzyme ($R_m = 0.14$) and the alkaline DNAase ($R_m = 0.065$)]. (3) Other sources of DNAases, such as serum, give complex patterns having many activity spots distributed two-dimensionally throughout the gel.

The use of pH extremes during electrophoresis makes possible the separation and detection of nuclease activities in the presence of the highly charged substrate without the use of SDS or other protein denaturants. Interaction of enzyme and substrate is minimized by using running pH values (and low temperature) at which enzyme activity is reversibly inhibited. A further advantage of using a running pH greater than the highest enzyme pI is that *all* enzyme activities migrate towards the anode. In addition, the net negative charge of the nucleases tend to repel them from the negatively charged substrate. However, if the pH is lower than the lowest pI, *all* the nucleases will move to-

Fig. 4. Effect of running and incubation conditions on the detection of DNAase activities

The supernatants from sonicated human leucocytes were subjected to isoelectric focusing, then electrophoresed: (a) 7.5×10^6 cells towards the anode at basic pH; (b and d) 1.5×10^6 cells towards the anode at basic pH; (c) 1.5×10^6 cells towards the cathode at acidic pH. After electrophoresis, gels were (a) incubated and stained to reveal the presence of protein; (b and c) incubated in 0.1 M-acetate buffer, pH 4.6, and stained to reveal the DNAase activities; (d) incubated in 100 mM-Tris/HCl, pH 7.4, containing 2.5 mM-MnCl₂ and 0.1 mM-CaCl₂ to locate other DNAase activities; (e) pH gradient.

wards the cathode. In the latter instance, movement of all positively charged proteins through the network of negatively charged DNA in the slab gel is not possible, owing to the tight electrostatic binding between polypeptide and polynucleotide. As described previously (Karpetsky *et al.*, 1980) this type of interaction may be overcome by titrating the negatively charged polynucleotide with an excess of a low-*M_r* polycation such as spermine, al-

though some streaking is sometimes observed (Figs. 4b and 4c).

Migration of enzymes towards the anode into the DNA-containing slab decreases with increasing protein isoelectric point when an alkaline running system is used (Fig. 4b). These relative rates of migration presumably are due to the net charge of the individual proteins increasing the further away their pI values are from the pH of the running buffer. As expected for acidic electrophoresis conditions (Fig. 4c), the rate of enzyme movement towards the cathode increases for higher pI values.

Incubation to activate nuclease activity

The incubation step makes isoelectric focusing-polynucleotide/polyacrylamide-gel electrophoresis particularly versatile. In a two-dimensional array of proteins (Fig. 4a), only certain enzymes are active under specific incubation conditions (Fig. 4b). Thus, from duplicate runs of a single sample, different classes of enzyme activity can be distinguished by varying the incubation conditions. For example, enzymes having an acid pH optimum (Fig. 4b) are distinct from those activities with a pH optimum near neutrality and having an obligatory requirement for bivalent cations found in the same sample (Fig. 4d). At pH 6.0, in the presence of metals, the gel pattern contains the activities visible in both Figs. 4(b) and 4(d). Furthermore, characterization of the pH and ionic-strength optima and substrate specificity of multiple enzymes is done easily by varying gel incubation conditions. An additional advantage of isoelectric focusing-polynucleotide/polyacrylamide-gel electrophoresis is its sensitivity, which permits the investigation of the nuclease content of very small samples. Not only are the contents of only $(1-1.5) \times 10^6$ cells per gel sufficient to obtain an enzyme pattern, but the individual isoenzymes of bovine pancreas DNAase I are detected at 50 pg of the individual enzyme species.

An important advantage of our system is that protein denaturants, such as SDS, do not have to be removed to re-activate enzyme activity. Although SDS can be removed from proteins in the gels using such treatments as incubation in large volumes of buffer (Rosenthal & Lacks, 1977) or propan-2-ol (Blank *et al.*, 1982), doubt always exists that *each* and *every* possible nuclease has been re-activated (Rabin & Weinberger, 1975; Blank & Dekker, 1981). Not only may the amount of activity recovered vary considerably with the lot of SDS, but undesirable additives to the gel, such as bovine serum albumin, may be necessary for optimal enzyme renaturation (Lacks *et al.*, 1979; Blank *et al.*, 1982). Furthermore, the information lost as a result of avoidance of SDS, i.e., accurate enzyme

M_r values, is more than compensated for by the availability of *native* enzyme for studies such as Ferguson analysis (Hedrick & Smith, 1968) of DNAase size and charge (Brown *et al.*, 1982) or analysis of charged carbohydrate moieties (see below).

Determination of isoelectric points

The isoelectric points of three enzyme activities present in purified bovine pancreatic DNAase I are easily determined to be 5.3, 5.1 and 5.0 using isoelectric focusing–polynucleotide/polyacrylamide-gel electrophoresis. These values are similar to those determined previously (Tournut *et al.*, 1978) using isoelectric focusing (5.29, 5.19 and 5.04). Comparisons of electrophoretic patterns obtained using DNAase I from five different commercial sources revealed that all preparations have similar multiple activities near pI 5.0. However, materials from Sigma and Miles also have significant DNAase activity at pI 6. Thus isoelectric focusing–polynucleotide/polyacrylamide-gel electrophoresis can be used to assign enzyme pI values with an accuracy similar to that obtained by traditional methods but with a minimum of sample manipulation.

Glycosidase-induced modification of DNAases

Examination of isoelectric-focusing–polynucleotide/polyacrylamide-gel electrophoresis gels for the presence of human intracellular DNAase II-like activities reveals an array of activities (Figs. 5a and 5b). It has been known for some time that there are multiple forms of a variety of types of human DNAases (Churchill *et al.*, 1973; Urbanczyk & Studzinski, 1974; Yamanaka *et al.*, 1974; Zollner *et al.*, 1974b, 1975a,b,c; Linsley *et al.*, 1977; Reimer *et al.*, 1978; Tournut *et al.*, 1978; Slor *et al.*, 1980). Since part of the variability of bovine and ovine pancreatic DNAases is due to variable oligosaccharide side chains (Salnikow *et al.*, 1970; Wadano *et al.*, 1979), a similar mechanism might be responsible for part of the pI heterogeneity we observe. Consequently, the crude DNAases were treated with various specific endo- and exo-glycosidases and then were examined for changes in enzyme activity patterns. Two-dimensional nuclease patterns do not change after incubation of samples in the absence of glycosidases for at least 72 h. Also, treatment of intracellular contents with several glycosidases (β -glucuronidase, β -galactosidase, xylosidase, α -glucosidase and α -mannosidase) for at least 72 h has no influence on DNAase activity patterns. However, the use of endo-H results in the complete disappearance of many activity spots having more acidic pI values (Fig. 5c). No new activities are detected after endo-H treatment. Furthermore, treatment of individual

Fig. 5. Simplification of DNAase activity patterns after glycosidase treatment

Enzyme treatments and isoelectric focusing–polynucleotide/polyacrylamide-gel electrophoresis were accomplished by using samples and gels prepared as described in the Materials and methods section. Gel (a) shows the pattern obtained from the sonicated intracellular contents of 4.5×10^6 hairy-cell leukaemic leucocytes. Gel (b) shows that incubation of the intracellular contents of 40×10^6 hairy-cell leukaemic leucocytes in 0.1 M-sodium acetate buffer, pH 5.8, at room temperature for 3 days, had no effect on enzyme pattern. Gel (c) shows that supplementing the reaction mixture with 0.2 unit of endo-H and incubating for 3 days resulted in simplification of patterns. Similarly, gel (d) shows changes in activities after treatment with 0.2 unit of *Vibrio cholera* neuraminidase for 3 h (or identical changes with 1.6 units of *Arthrobacter ureafaciens* neuraminidase for 3 h). Gel (e) shows a different type of simplification found after incubation with 60 units of *Clostridium perfringens* neuraminidase for 3 h (pH gradients are displaced 12 mm to the left with respect to the gradient shown in Fig. 4). All gels show the patterns derived from the contents of 4.5×10^6 cells.

species of DNAase isolated on the basis of pI by Chromatofocusing (Pharmacia) show a conversion of enzyme activity from a relatively acidic pI into one at a relatively alkaline pI (results not shown). Thus the pI values for certain individual intracellular DNAases increase as a consequence of endo-H-induced loss of negative charge, possibly owing to the loss of the negatively charged sugar *N*-acetylneuraminic acid.

Although neuraminic acid generally is not found on endo-H-sensitive structures, it has been reported on complex hybrid-branched structures that

retain sensitivity to endo-H (Hunt & Wright, 1981). Accordingly, the human DNAases were incubated with proteinase-free neuraminidase from *Clostridium perfringens*, *Vibrio cholera* and *Arthrobacter ureafaciens*. Multiple activity spots disappear as a consequence of neuraminidase action (Figs. 5d and 5e). Some of these activities are the same as those that disappear on endo-H treatment. However, some of the spots having pI values of 6.45 and 6.35 are resistant to neuraminidase action, even though they become more positively charged after endo-H treatment. In addition, there appears to be heterogeneity in the moieties containing neuraminic acid, since there is a difference in results between the three neuraminidases used. For example, only the *Clostridium* enzyme causes one of the neuraminidase-resistant spots to diminish over long incubations. The partial resistance to neuraminidase action might be due to steric hindrance resulting from branching at the residue adjacent to *N*-acetylneuraminic acid (Gottschalk & Drzeniek, 1972) or to the presence of neuraminic acid derivatives with various sensitivities to the neuraminidases employed. Activities resistant to both endo-H and neuraminidase may represent multiple polypeptide species or enzyme bearing other types of charged modifications.

N-Glycosidic carbohydrate-peptide linkages have been found in other nucleases, such as bovine pancreatic ribonuclease B (one/molecule; Plummer & Hirs, 1963), porcine pancreatic ribonuclease (three/molecule; Jackson & Hirs, 1970a,b), other mammalian RNAases (Beintema *et al.*, 1976), and bovine pancreatic DNAase (Catley *et al.*, 1969). Furthermore, *N*-acetylneuraminic acid appears to be present on certain of the neutrophil DNAases and thus is responsible for some of the heterogeneity of charge. Thus some of the DNAase activities appear to contain an oligomannose core, characteristic of neutral structures, but containing terminal *N*-acetylneuraminic acid residues more frequently associated with complex acidic structures. Previously, hybrid carbohydrates of this type have been found only in Rous-sarcoma-virus glycoproteins (Hunt & Wright, 1981), although other types of hybrid structures have been identified in other glycoproteins (Tai *et al.*, 1977a,b; Yamashita *et al.*, 1978; Kobata, 1979).

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