RESEARCH COMMUNICATION

Immobilized pH gradient focusing of alkaline proteins: analysis of the isoform composition of purified human non-secretory ribonucleases from kidney, liver and spleen

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Previous studies on the isoform composition of human ribonucleases (RNAases) have resulted in confusing and inconsistent results, presumably due to methodological problems in electrofocusing of alkaline proteins. In the present study, immobilized pH gradient (IPG) carrier ampholyte (CA) isoelectric focusing (IEF) and conventional CA-IEF have been evaluated for the analysis of the isoforms of human non-secretory RNAases purified from kidney, liver and spleen. CA-IEF proved unsuitable since the alkaline RNAase isoforms migrated into the cathode. IPG-CA-IEF, however, resolved the RNAase isoforms and

marker proteins in the basic region of the gel matrix. The three RNAases had comparable isoform profiles, each with two protein bands with approximate pI values of 10.3 and 10.4. Western blotting showed that the two protein bands of each RNAase were immunoreactive (with polyclonal antibodies that recognize RNAase), indicating that the protein bands are RNAase isoforms. The present results provide reliable pI data on human RNAase isoforms and suggest that IPG-CA-IEF should be a suitable technique for analysing the isoforms of other alkaline proteins.

INTRODUCTION

Interest in ribonucleases (RNAases) has been rekindled by findings indicating that a large number of proteins with diverse functions belong to a 'superfamily' with substantial sequence identity with RNAases. Included among these proteins are angiogenin, a protein secreted by tumours which induces new blood vessel growth [1]; P-30, an anti-tumour protein isolated from eggs and early embryos [2]; and bovine seminal RNAase, an anti-tumour and immunosuppressive protein isolated from bovine seminal fluid [3]. A number of proteins in this superfamily are cytotoxic, including bovine RNAase A [4] and two proteins derived from human eosinophils, eosinophil-derived neurotoxin (EDN) [5] and eosinophil cationic protein (ECP) [6]. EDN is a potent neurotoxin while ECP is a potent helminthotoxin. Angiogenin, P-30, EDN and ECP all have measurable RNAase activity. Investigators have also been interested in RNAases with regard to alterations of their amounts, activities and properties in a number of human diseases including cancer (reviewed in [7]).

In previous studies we purified non-secretory RNAases from human kidney, liver and spleen and characterized their kinetic, peptide mapping and lectin blotting properties [8], as well as the structures of their N-glycans by ¹H-n.m.r. spectroscopy and electrospray mass spectrometry [9]. The results of these studies have indicated that the three human tissue RNAases are very similar, if not identical, in all of the properties investigated. All three RNAases have the same N-glycans, which may vary slightly in relative amounts in the different tissues [9] and which contribute to the microheterogeneity of these glycoproteins.

In the present study, the isoform compositions of the three purified human non-secretory RNAases have been investigated. Previous isoelectric focusing (IEF) studies on human RNAases have used carrier ampholytes (CAs) in slab gels or columns and

have given inconsistent and confusing results (see the Results and discussion section). The unreliability of these results appears to be due to methodological problems such as cathodic drift [10], which is particularly troublesome in analysing proteins with high pI values such as the alkaline RNAases. These proteins with high pI values accumulate near and migrate into the cathodic wick. In an attempt to circumvent this problem, we have employed the immobilized pH gradient (IPG) technique introduced by Bjellqvist et al. [11] for the first time to analyse human RNAases. The results of the current studies indicate that the IPG technique is useful for studying human tissue RNAases and that all three RNAases have comparable isoform profiles, with at least two immunoreactive isoforms with pI values of approximately 10.3 and 10.4. In addition, the results suggest that the IPG technique should be useful for analysing the isoform composition of other alkaline proteins.

EXPERIMENTAL

Purification of RNAases

Non-secretory RNAases were purified to apparent homogeneity from human kidney, liver and spleen using a six-step procedure (acid denaturation, heat denaturation, cation-exchange chromatography, Affi-Gel-heparin chromatography, agarose-5'-(4-aminophenylphosphoryl)uridine 2'(3')-phosphate chromatography and size-exclusion chromatography) as previously described in detail [8].

IEF of RNAases

Standard CA-IEF was performed essentially as described by Righetti [12] using 7% polyacrylamide slab gels (0.75 mm), 2.5% (w/v) pH 9–11 Servalyt (CA) (Serva, Hauppauge, NY,

Abbreviations used: CA, carrier ampholyte; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; IEF, isoelectric focusing; IPG, immobilized pH gradient; PVDF, poly(vinylidene difluoride); TEMED, *NNN'*-tetramethylethylenediamine.

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Table 1 Composition of the light and dense gel solutions for IPG-CA-IEF

Each procedure gives 15 ml, which can be used to make two slab gels.

Components	Acid light solution (pH 8)	Basic dense solution (pH 11)
18 mΩ/cm water	3.0 ml	1.0 ml
Immobiline pK 7.0	364 μl	311.5 μl
Immobiline pK 8.5	355 μl	371.5 μl
Immobiline pK 9.3	94 μl	388.5 μl
Immobiline pK 3.6	399 μl	—
0.5/0.05 M Formic acid	Adjust to pH 7.0	Adjust to pH 7.0
Servalyt pH 7–9 (40%, w/v)	100 μl	100 μl
Servalyt pH 9–11 (40%, w/v)	100 μl	100 μl
Stock acrylamide (T30C4)	3.5 ml	3.5 ml
Sorbitol (60%, w/v)	2.0 ml	7.5 ml
18 mΩ/cm water	Adjust to 15 ml	Adjust to 15 ml

U.S.A.) and 30 % (w/v) sorbitol (Sigma Chemical Co., St. Louis, MO, U.S.A.). Gels were photopolymerized with a fluorescent lamp using 0.05 % (w/v) riboflavin (Sigma), 0.015 % (w/v) ammonium persulphate (Bio-Rad, Hercules, CA, U.S.A.) and 0.05 % (w/v) *NNN'*-tetramethylethylenediamine (TEMED) (Bio-Rad) in the gel solutions. Gels were prefocused at 500 V for 20 min and focusing was done at 7 W (constant power) for 2.5 h at 10 °C with an inter-electrode distance of 11 cm. Gels were stained/destained as described by Patestos et al. [13] using Violet 17 (Serva).

Slab gel IPG-CA-IEF was performed essentially as described [14,15]. The amounts of Immobililine II (Pharmacia-LKB, Piscataway, NJ, U.S.A.) solutions used for gel preparation were calculated by extrapolation of the protocol for a broad pH range 8–10 (Table 2.3 in ref. [15]), modified to extend the basic end point of the pH gradient to 11 (see Table 1). The order of addition of the components, as shown in Table 1 starting at the top and moving to the bottom, is important in order to prevent destruction of Immobililines and to allow gel polymerization to occur. The Immobililines were put in 18 mΩ/cm water and the pH was adjusted to 7.0 with 0.5 M and/or 0.05 M formic acid. The Servalyts, pH 7–9 and 9–11 (Serva), acrylamide (Bio-Rad) and sorbitol (Sigma) were added and brought to 15 ml with 18 mΩ/cm water. The acidic light solution and basic dense solution (Table 1) were each split into two 7.5 ml portions, each of which is sufficient for one gel (140 mm × 140 mm × 0.85 mm) (see Table 1).

Slab gels were cast according to the manufacturer's instructions using a two-chamber Hoefer SG 30 gradient maker at a flow rate of approx. 2 ml/min into a Hoefer SE 6015 gel caster consisting of a plain glass plate (16 cm × 18 cm) which had been coated with Repel-Silane (Pharmacia-LKB), an untreated notched glass plate (16 cm × 18 cm) and 0.75 mm spacers. Basic dense solution (2 ml) was added first to create a pH 11 plateau [after adding 0.6 μl of TEMED and 2 μl of 40 % (w/v) ammonium persulphate]. TEMED (3.7 μl) and 7.5 μl of 40 % (w/v) ammonium persulphate were added to the acidic light solution, and 2.2 μl of TEMED and 7.5 μl of ammonium persulphate were added to the basic dense solution just prior to casting of the gel. The gels were polymerized at 50 °C for 2.5 h, cooled at room temperature for 1.5 h and cooled at 10 °C for 40 min on a cooling plate.

Electrode wicks (Whatman 3M paper) were impregnated with 0.25 M Hepes (Sigma) for the anode and 1.0 M NaOH for the cathode. Protein samples (3–8 μg per lane) were applied in two

places, 1 cm and 3 cm from the anode, so that the equilibrium position for each protein could be determined. Horse heart cytochrome *c* (Sigma) and chicken egg white lysozyme (grade VI; Sigma) were run at high-pI protein markers. Electrofocusing was done without prefocusing over an inter-electrode distance of 11 cm for 12 h at 10 °C using a VWR EC-600 power supply set with upper limits of 300 V, 5 W and 5 mA. After electrofocusing, the gels were immersed in 400 ml of fixing solution (20 % trichloroacetic acid) (Fisher Scientific, Pittsburg, PA, U.S.A.) for 60 min followed by washing in 3 % H₃PO₄ for 5 min. Gels were stained/destained as described [13] with colloidal Violet 17 (Serva), immersed in preserving solution [2.5 % (w/v) glycerol] for 5 min and placed on Gelbond PAG film (FMC, Rockland, ME, U.S.A.) to support the gel during drying at room temperature in the dark.

Western immunoblotting of RNAases

Western blotting of IPG-CA-IEF gels was done essentially as described by Cowdrey et al. [14]. The dried gels were destained completely in 3 % (w/v) H₃PO₄ for 12 h, rinsed with a large volume of 18 mΩ/cm water, and proteins were transferred to a poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad) that had been prewashed with methanol for 2–3 s and with 18 mΩ/cm water for 15 min, then equilibrated in 0.01 M PBS, pH 7.2. Capillary transfer of proteins was carried out for 1 h at 10 °C, after which the membrane was blocked in a solution of 1 % (w/v) dried non-fat milk (Carnation) in PBS for 1 h with constant shaking at room temperature. Immunodetection was carried out with anti-(human EDN) [16] (generously provided by Dr. G. J. Gleich, Mayo Clinic, Rochester, MN, U.S.A.) diluted 1:1250 with 0.2 % (w/v) dried non-fat milk in PBS for 2 h. The membrane was washed three times over 5 min in PBS and incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit IgG (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) diluted 1:1000 in 2 % (w/v) dried non-fat milk in PBS. The membranes were re-washed three times over 5 min in PBS, stained for colour for 10–15 min in a solution of 50 μl of 60 mM Nitro Blue Tetrazolium (Bio-Rad), 37.5 μl of 120 mM 5-bromo-4-chloroindolyl phosphate (Bio-Rad) and 10 ml of 0.1 M Tris/HCl, pH 9.5, containing 0.1 M NaCl and 5 mM MgCl₂, washed in 18 mΩ/cm water, and dried at room temperature.

RESULTS AND DISCUSSION

A number of investigators have used either preparative IEF for purifying human RNAases [5,17] or analytical IEF for characterizing human secretory [18–21] and non-secretory [19,20] RNAases. All of these studies have used conventional CAs in either columns [17,19] or slab gels [18,20,21]. The results of these studies have not been very definitive, and RNAase pI values are given only as a broad range, and in some cases are not given at all. In general, the results have indicated that both secretory and non-secretory RNAases exist in multiple isoforms, primarily with neutral-to-alkaline pI values. The inconsistent and confusing results appear to be due, at least in part, to methodological problems associated with IEF of alkaline proteins, which tend to migrate into the cathode compartment. This cathodic drift appears to be inherent to the technique of IEF when using CAs [10]. The introduction of IPGs [11] has led to stable pH gradients, due to the covalent attachment of ampholytes (Immobililines) to the gel matrix, which minimizes cathodic drift. CAs are also included when IPG gels are used to decrease hydrophobic interactions between the proteins being focused and the IPG matrix [15].

Figure 1 IPG-CA-IEF of purified human non-secretory RNAases

Lanes 1 and 10, horse heart cytochrome *c* (3 µg/lane); lanes 2 and 9, chicken egg white lysozyme (3 µg/lane); lanes 3 and 6, kidney RNAase (6 and 8 µg/lane respectively); lanes 4 and 7, liver RNAase (6 and 8 µg/lane respectively); lanes 5 and 8, spleen RNAase (6 and 8 µg/lane respectively). See the Experimental section for further details.

In the present investigation, IPG-CA-IEF has been employed for the first time to analyse the isoforms of human RNAases. More specifically, the isoforms of non-secretory RNAases purified to apparent homogeneity from human kidney, liver and spleen in a previous study [8] have been analysed by both slab gel CA-IEF and IPG-CA-IEF. As expected, the former technique proved to be unsuitable since the alkaline human RNAases migrated into the cathode compartment (results not shown). The protocol for IPG-CA-IEF of the human RNAases was modified from the published procedure for pH range 8–10 (Table 2.3 in ref. [15]) to extend the basic range to pH 11, since Immobiline pK 10.3 (which is necessary for generating a pH 8–11 gradient) is not currently commercially available (see Table 1). The results of IPG-CA-IEF of the RNAases between pH 8 and approx. 10.7 (Figure 1) indicated the presence of two protein bands for kidney (lanes 3 and 6), liver (lanes 4 and 7) and spleen (lanes 5 and 8) RNAases when samples of either 6 µg/lane (lanes 3–5) or 8 µg/lane (lanes 6–8) were focused. Initial focusing of the human RNAases without the pH 11 plateau caused these basic proteins to migrate very close to the cathodic wick, and lysozyme drifted into the cathode (results not shown). Addition of the plateau to the basic end of the gel maximized the use of the basic end of the pH gradient, prevented lysozyme from migrating into the cathode and kept the RNAases at a safe distance from the cathode (Figure 1). The co-migration of the two sets of samples (lanes 1–5 and 6–10) loaded 2 cm apart suggests that the equilibrium positions of the proteins have been achieved. Cytochrome *c* and lysozyme, with reported pI values of 10.25 and 10.6 respectively when subject to IPG-CA-IEF [14,15], were run on the gel (lanes 1, 2, 9 and 10) in order to estimate the pIs of the human RNAases. This was done because surface pH electrode determinations on IPG matrices are unreliable due to the low conductivity of these gels [22]. Each human RNAase had two isoforms, with approximate pIs of 10.3 and 10.4, running between cytochrome *c* and lysozyme. When all three RNAases were loaded in one lane they gave the same isoform pattern as when each RNAase was loaded in individual lanes (results not shown), further suggesting that the RNAases have comparable isoform compositions.

Western immunoblotting of lanes 6–8 of Figure 1 was carried out to determine if both protein bands for each RNAase were recognized by a polyclonal antibody that recognizes human non-

Figure 2 Western immunoblot of purified human non-secretory RNAases after IPG-CA-IEF

Lanes 6, 7 and 8 from Figure 1 were blotted on a PVDF membrane and detected with polyclonal antibodies against EDN, which is homologous to human non-secretory RNAase [16]. See the Experimental section for further details.

secretory RNAase [16]. The IPG-CA-IEF gel was sliced horizontally between the two protein bands in each lane prior to blotting so that it would be easier to interpret the blotting results of closely migrating bands. Figure 2 shows that both protein bands for kidney (lane 6), liver (lane 7) and spleen (lane 8) RNAases were immunoreactive, indicating that both protein bands represent RNAase.

The results of the present study indicate that kidney, liver and spleen non-secretory RNAases are similar in another property and have comparable isoform profiles, each with two isoforms at approximate pI values of 10.3 and 10.4. Previous CA-IEF studies on non-secretory RNAases derived from urine have provided less definitive results, and suggested the presence of three isoforms with pI values of > 9 [18] and one isoform of pI > 11 [19]. The comparative analysis of RNAases (and cytochrome *c* and lysozyme) using both CA-IEF and IPG-CA-IEF in the present study also indicates that the latter technique is far superior and more reliable than the former technique for analysis of these proteins. Our results suggest that IPG-CA-IEF should be useful for the analysis of other alkaline proteins.

This research was supported in part by a grant from the Dorothy Rider Pool Health Care Trust. We acknowledge Dr. Cris W. Lawrence for technical advice and Mrs. Dolores Weaver for excellent secretarial help.

REFERENCES

- 1 Strydom, D. J., Fett, J. W., Lobb, R. R., Alderman, E. M., Bethune, J. L., Riordan, J. F. and Vallee, B. L. (1985) *Biochemistry* **24**, 5486–5494
- 2 Ardelt, W., Mikulski, S. M. and Shogen, K. (1991) *J. Biol. Chem.* **266**, 245–251
- 3 D'Alessio, G., DiDonato, A., Parente, A. and Piccoli, R. (1991) *Trends Biochem. Sci.* **16**, 104–106
- 4 Rybak, S. M., Saxena, S. K., Ackerman, E. J. and Youle, R. J. (1991) *J. Biol. Chem.* **266**, 21202–21207
- 5 Beintema, J. J., Hofsteenge, J., Iwama, M., Morita, T., Ohgi, K., Irie, M., Sugiyama, R. H., Schieven, G. L., Dekker, C. A. and Glitz, D. G. (1988) *Biochemistry* **27**, 4530–4538
- 6 Barker, R. L., Loegering, D. A., Ten, R. M., Hamann, K. J., Pease, L. R. and Gleich, G. J. (1989) *J. Immunol.* **143**, 952–955
- 7 Maor, D. and Mardiney, M. R. (1979) *CRC Crit. Rev. Clin. Lab. Sci.* **10**, 89–111
- 8 Lawrence, C. W., Little, P. A., Little, B. W., Miller, M. J., Bazell, S. and Alhadeff, J. A. (1993) *Glycobiology* **3**, 241–248
- 9 Lawrence, C. W., Little, P. A., Little, B. W., Glushka, J., Van Halbeek, H. and Alhadeff, J. A. (1993) *Glycobiology* **3**, 249–259
- 10 Righetti, P. G. and Drysdale, J. W. (1973) *Ann. N.Y. Acad. Sci.* **209**, 163–186
- 11 Bjellqvist, B., Ek, K., Righetti, P. G., Gianazza, E., Gorg, A., Postel, W. and Westermeier, R. (1982) *J. Biochem. Biophys. Methods* **6**, 317–339
- 12 Righetti, P. G. (1983) *Isoelectric Focusing: Theory, Methodology and Applications*, Elsevier, Amsterdam
- 13 Patestos, N. P., Fauth, M. and Radola, B. J. (1988) *Electrophoresis* **9**, 488–496
- 14 Cowdrey, G., Gould, B., Rees, J. and Firth, G. (1990) *Electrophoresis* **11**, 813–818

- 15 Righetti, P. G. (1990) *Immobilized pH Gradients: Theory and Methodology*, Elsevier, Amsterdam
- 16 Gleich, G. J., Loegering, D. A., Bell, M. P., Checkel, J. L., Ackerman, S. J. and McKean, D. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3146–3150
- 17 Kurihara, M., Ogawa, M., Ohta, T., Kurokawa, E., Kitahara, T., Kosaki, G., Watanabe, T. and Wada, H. (1982) *Cancer Res.* **41**, 4836–4841
- 18 Yasuda, T., Nadano, D., Tenjo, E., Takeshita, H. and Kishi, K. (1992) *Anal. Biochem.* **206**, 172–177
- 19 Cranston, J. W., Perini, F., Crisp, E. R. and Hixson, C. V. (1980) *Biochim. Biophys. Acta* **616**, 239–258
- 20 Mizuta, K., Yasuda, T., Ikehara, Y., Sato, W. and Kishi, K. (1990) *Z. Rechtsmed.* **103**, 315–322
- 21 Yasuda, T., Nadano, D., Tanaka, Y. and Kishi, K. (1992) *Biochim. Biophys. Acta* **121**, 331–334
- 22 Righetti, P. G., Gianazza, E., Gelfi, C. and Sinha, P. K. (1986) in *Electrophoresis '86* (Dunn, M., ed.), pp. 419–434, VCH, Weinheim

Received 24 September 1993; accepted 18 October 1993