ELSEVIER

Journal of Chromatography A, 772 (1997) 305–311

JOURNAL OF CHROMATOGRAPHY A

Identification of α_1-acid glycoprotein (orosomucoid) in human synovial fluid by capillary electrophoresis

Aislinn Rice, James Grimshaw*, Jadwiga Trocha-Grimshaw, Philip McCarron, G. Brian Wisdom

School of Chemistry and School of Biology and Biochemistry, The Queen's University of Belfast, Belfast BT9 5AG, Northern Ireland, UK

Abstract

Capillary electrophoresis of human synovial fluid in a phosphate–borate run buffer containing sodium dodecyl sulphate separates a hydrophilic glycoprotein, hyaluronan and a number of low-molecular-mass components. The hydrophilic glycoprotein is identified as α_1-acid glycoprotein (AGP), orosomucoid, by co-injection methods with human AGP and by reaction with neuraminidase which released N-acetylneuraminic acid. Finally, a sample of the glycoprotein was isolated by micropreparative capillary electrophoresis, examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis methods and shown to give a positive reaction with AGP antibodies. The peak due to AGP in the capillary electrophoresis is broad and gives evidence for the presence of glycoforms.

Keywords: α_1-Acid glycoprotein; Glycoproteins; Proteins

1. Introduction

α_1-Acid glycoprotein (AGP) belongs to the group of proteins referred to as positive acute phase proteins. Human AGP (M_r $41 \cdot 10^3$) is readily isolated from pooled samples of plasma. The complete sequence of 181 amino acid residues in the protein moiety has been determined [1]. The pooled sample examined showed 20 positions for amino acid substitutions. Six of these substituting pairs involved phenylalanine and a second aliphatic amino acid, while one involved the tyrosine leucine pair. Assuming half a residue at each substitution, the total residues of aromatic amino acids in one molecule of AGP are Phe-8, Try-11, Trp-3. Carbohydrate units are attached to this protein core at five points by N-glycosylation to asparagine [2]. These units are very heterogeneous [3,4]. Approximately 90% of the sugar chains contain more than three outer branches, many of which are terminated by N-acetylneuraminic acid (sialic acid) residues attached through α-glycosidic bonds to the 3- or 6-position in the penultimate residue of galactose [5].

Thus AGP shows considerable heterogeneity resulting from amino acid substitutions, variations in the five oligosaccharide chains and from the extent of sialylation. Many studies which utilised the technique of affinity electrophoresis with concanavalin-A have demonstrated the micro-heterogeneity of AGP derived from serum and from synovial fluid in disease states [6–9]. Recently, methods have been developed for the isolation of AGP with negligible degradation, so that the heterogeneity of the molecule can be probed further [10]. The significance of AGP levels in both synovial fluid and serum in disease states has been discussed by various authors

*Corresponding author.

0021-9673/97/$17.00 © 1997 Elsevier Science B.V. All rights reserved
PII S0021-9673(96)00990-9

[11,12]. The identification and quantification of AGP in synovial fluid by capillary electrophoresis may provide a useful indicator for diagnosis in the early stages of various types of arthritis.

The identification of the peak due to AGP from capillary electrophoresis of both human synovial fluid and serum is demonstrated in this paper. This is the peak which we have labelled b in previous publications on the capillary electropherograms of synovial fluid [13,14], and a procedure was developed for the quantification, in terms of an arbitrary unit, of the compound responsible for this peak [15].

2. Experimental

2.1. Synovial fluid samples

Samples were obtained by aspiration, either during routine clinical investigation under local anaesthetic or during total knee joint replacement surgery under general anaesthetic, and immediately plunged into liquid nitrogen. Full clinical details were available with all samples. The samples were thawed and centrifuged for 1 h at 15 000 *g* prior to enzymatic treatment.

2.2. Buffers and enzymes

2.2.1. CE carrier buffer

The buffer (pH 9) contained 50 m*M* disodium hydrogenphosphate, 10 m*M* sodium tetraborate and 40 m*M* sodium dodecyl sulphate (SDS). It was used in our previous studies with synovial fluid [13,14].

2.2.2. Neuraminidase reagent

Neuraminidase (EC 3.2.1.18), recombinant form derived from *Salmonella typhimurium*; was purchased from Sigma (Sigma–Aldrich, Poole, UK). The enzyme (5 units) was diluted with 500 μl of a buffer containing 67 m*M* potassium dihydrogenphosphate adjusted to pH 5.5 with 67 m*M* disodium hydrogenphosphate. The solution was divided into 100 μl aliquots and kept at −20°C until required.

2.2.3. N-Acetylneuraminic acid aldolase reagent

N-Acetylneuraminic acid aldolase (EC 4.1.3.3) from *Clostridium perfringens* was purchased from Sigma. The enzyme (1 unit) was dissolved in 200 μl of 67 m*M* phosphate buffer pH 7.2 containing 7.0 ml 67 m*M* disodium hydrogenphosphate and 3.0 ml 67 m*M* potassium dihydrogenphosphate. The solution was divided into 20 μl lots and kept at −20°C until required. The thawed enzyme was diluted with a further 100 μl of buffer, pH 7.2, and kept at 5°C before use.

2.2.4. Gel electrophoresis and immunoblotting reagents

AGP antibody was purchased from The Binding Site (Birmingham, UK), sheep IgG antibody/alkaline phosphatase from Sigma and molecular mass standards were purchased from Bio-Rad (Hercules, CA, USA).

Sample treatment buffer: 0.125 *M* Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.001% bromophenol blue plus pyronin-Y as the dyes. Electrophoresis buffer (pH 8.3): 0.025 *M* Tris, 0.192 *M* glycine, 0.1% SDS and 10% methanol. Transfer buffer (pH 8.3): 0.025 *M* Tris, 0.192 *M* glycine, containing 10% methanol. Blocking solution: 5% dried milk powder in Tris-buffered saline. Tris-buffered saline (pH 7.4): 20 m*M* Tris–HCl containing 0.15 *M* sodium chloride.

2.3. Conditions for capillary electrophoresis

A Beckman P/ACE 5000 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) was used with a 75 μm diameter fused-silica capillary, overall length 58.7 cm, length to detector 50 cm. The capillary temperature was maintained at 30°C. Pressure injection at the anodic end was used. Routine detection was by on-column measurement of ultraviolet absorption at 200 nm, column length to detector window 50 cm. In some cases the UV spectra of eluting materials were obtained using a Beckman diode array detector attached to the column window. Results were recorded on a computer with Beckman System Gold software at a frequency of 5 Hz.

2.3.1. Analytical mode

Between runs and at the start of a sequence, the capillary was washed in succession with 0.1 m*M*

sodium hydroxide solution and water, using nitrogen pressure for 2 min each. The capillary was flushed with the carrier buffer for 2 min prior to an analytical run. Samples were loaded by applying nitrogen pressure for 5 s, after which separation was performed by application of a potential of 15 kV for 20 min.

2.3.2. Collection mode

Between runs and at the start of a sequence, the capillary was washed in succession with 0.1 m*M* hydrochloric acid, 0.1 m*M* sodium hydroxide and water, using nitrogen pressure for 5 min each. The capillary was then flushed with the carrier buffer for 2 min prior to a run. Undiluted synovial fluid was loaded by applying nitrogen pressure for 10 s and separation conducted by application of a potential of 15 kV. Just prior to the leading edge of the AGP peak reaching the detector window, the potential was removed and the capillary exit placed in a collection vial containing 5 μl of water. Separation was continued under a potential of 5 kV and noting when the trailing edge of the peak passed the detector window. Separation was continued for the time calculated for this peak to traverse the 7.5 cm to the end of the capillary, after which the potential was removed and the capillary exit placed in the original exit vial ready for a further separation. The peak velocity at 5 kV is calculated from the measured velocity at 15 kV. After a preliminary run to determine conditions, the sequence can be set to repeat automatically.

2.4. Identification of AGP

2.4.1. Co-injection method

Human AGP prepared from Cohn fraction VI was purchased from Sigma. A solution containing 1 mg/ml was prepared in water. A sample of synovial fluid (10 μl), from a patient with inflammatory joint disease, was examined by the analytical capillary electrophoresis method. A mixture of this fluid (10 μl) with the AGP solution (10 μl) was then examined under the same conditions.

2.4.2. Enzyme degradation methods

N-Acetylneuraminic acid (synthetic crystalline) was purchased from Sigma and used as a 1 mg/ml solution in water. An undiluted sample synovial fluid from a patient with inflammatory joint disease was analysed by capillary electrophoresis. A 30 μl sample of the same synovial fluid was then added to an equal volume of neuraminidase solution and incubated at 37°C for 5 h. A 10 μl sample of this solution was analysed by capillary electrophoresis. The remaining solution was centrifuged at 5000*g* for 40 min through a size-exclusion filter (M_r 5000) purchased from Millipore (UK), Watford, UK. One 10 μl sample of the filtrate was examined by capillary electrophoresis for evidence of released N-acetylneuraminic acid and a further sample was co-injected with N-acetylneuraminic acid solution. A third 10 μl sample was mixed with 10 μl of the N-acetylneuraminic acid aldolase solution and incubated for 1 h at 37°C. The products were analysed by capillary electrophoresis.

2.4.3. Preparative capillary electrophoresis, polyacrylamide gel electrophoresis and immunoblotting methods

The synovial fluid sample utilised was obtained from a patient with definitive systemic lupus erythematosis, a rheumatic disease characterised by highly elevated levels of AGP. A minimum of 12 consecutive preparative capillary electrophoresis runs were collected, during which sequence the time width of the collection window at 5 kV was altered as required to allow for shifts in migration time due to gradual protein coating of the capillary wall [17,18]. Some evaporation of the collected sample occurred and the volume was adjusted with water to 10 μl.

Polyacrylamide 5–15% linear gradient gels with 4% stacking gels were prepared according to the technique of Laemmli [19]. Samples were diluted with an equal volume of treatment buffer, boiled for 5 min and transferred to the gel. Electrophoresis was continued with a current of 30 mA for 60 min. The separated proteins were transferred from the gel to a nitrocellulose membrane (Schleicher and Schüll, Dassel, Germany) soaked in transfer buffer by application of 80 mA for 60 min. The membrane was incubated with the blocking solution for 1 h, rocked in a solution of 10 μl AGP-antibody in 5 ml blocking solution for 2 h, then rinsed (5×) with Tris-buffered saline for 30 min. Next, the membrane was rocked with a solution of 2 μl sheep anti-IgG/

alkaline phosphatase in 5 ml blocking solution for 2 h and rinsed with Tris-buffered saline as before. Finally the membrane was immersed in a solution of alkaline phosphatase substrate to reveal the AGP bands. Molecular masses were determined by comparison with prestained standards (Bio-Rad).

3. Results and discussion

A typical capillary electropherogram from a sample of synovial fluid taken from a patient with osteoarthritis is shown in Fig. 1. Peak d has been previously [14] identified as due to uric acid by co-injection and by UV-absorption characteristics. Peaks b and c are present in all samples of synovial fluid and vary considerably in area, depending on the disease state of the patient. Peak c was identified [14] as due to the glycosaminoglycan polymer, hyaluronan, by co-injection with an authentic sample and by degradation with hyaluronidase (EC 2.2.1.35) to give a characteristic ladder of peaks due to oligomers, partly resolved by capillary electrophoresis.

The species responsible for peak b shows a UV-absorption spectrum (Fig. 2), indicating the presence of a protein structure containing aromatic amino acids. Under our experimental conditions for electrophoresis in a buffer containing SDS, most of the proteins in synovial fluid are incorporated into the SDS micelles and give rise to the peaks in the migration time (t_m) region of 20–30 min. The compounds which gave rise to peaks in this region were degraded by papain (EC 3.4.22.2) [14]. The protein species giving rise to the peak b which has a much shorter migration time cannot be strongly incorporated into these micelles and must be more hydrophilic relative to other protein species. The first clue to the identity of the species responsible for peak b came when samples of serum were examined by capillary electrophoresis and a peak with the same migration time was also found to be prominent. AGP is a known component of both serum and synovial fluid, so the remainder of this work concentrated on establishing that AGP is responsible for peak b. Co-injection of synovial fluid and a commercial sample of human AGP increased the area under peak b (Fig. 3). Samples of synovial fluid were next treated in succession with neuraminidase and N-acetylneuraminic acid aldolase, and reactions followed by capillary electrophoresis (Figs. 4 and 5).

Purine degredation products and aromatic amino acids are present in sufficient quantities in body

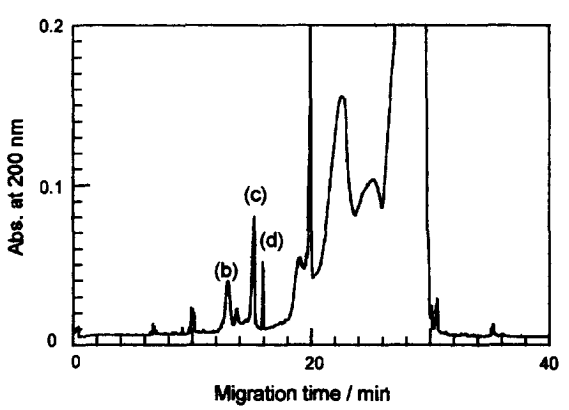

Fig. 1. Capillary electropherogram of human synovial fluid (from a patient with osteoarthritis). A 75 cm (50 cm to detector)×75 μm fused-silica capillary was used. The running buffer was 50 m*M* disodium hydrogenphosphate, 10 m*M* sodium tetraborate and 40 m*M* SDS, running voltage 15 kV (outlet negative). Identification of peaks: (b) AGP discussed in this paper, (c) hyaluronan, (d) uric acid.

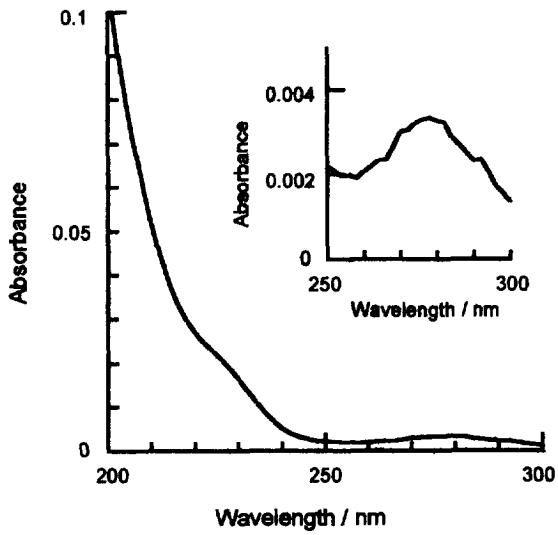

Fig. 2. Absorbance spectrum associated with peak (b) in Fig. 1. Data was collected by continuous monitoring of the eluent passing the capillary window using a diode array detector. This spectrum was obtained at the mid-point of peak (b). Inset has ordinate expanded ×10.

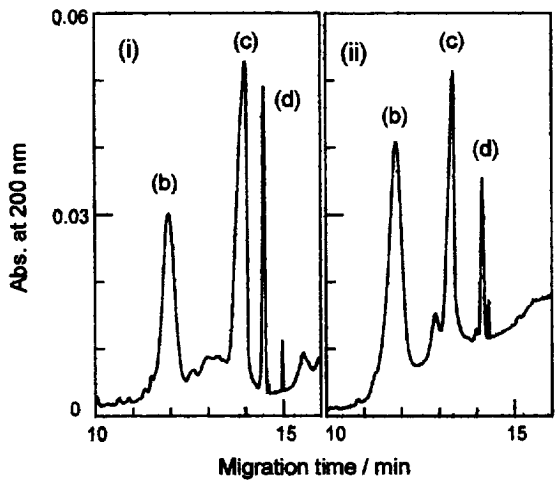

Fig. 3. Capillary electropherogram of human synovial fluid (from a patient with osteoarthritis). A 75 cm (50 cm to detector)×75 μm fused-silica capillary was used. The running buffer was 50 m*M* disodium hydrogenphosphate, 10 m*M* sodium tetraborate and 40 m*M* SDS, running voltage 15 kV (outlet negative). (i) Raw fluid. (ii) Fluid mixed with an equal volume of 1 mg/ml human AGP solution. Peak identification as in Fig. 1. Peak (b) is augmented by the addition, peaks (c) and (d) are diminished by the dilution.

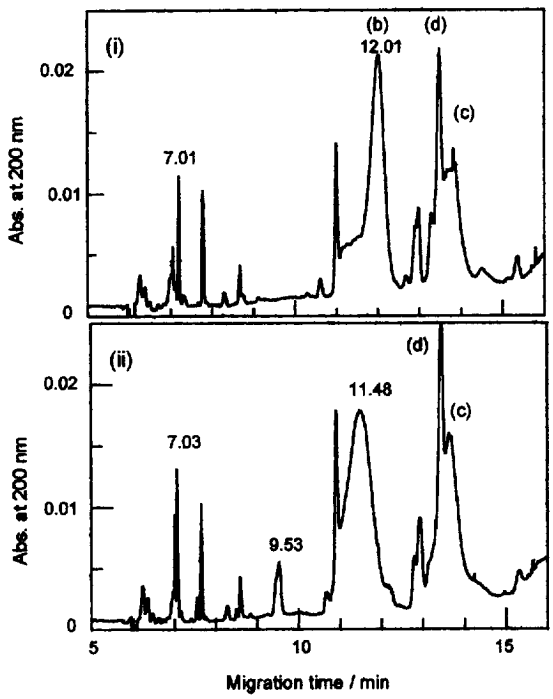

Fig. 4. Capillary electropherogram of human synovial fluid (from a patient with rheumatoid arthritis). A 75 cm (50 cm to detector)× 75 μm fused-silica capillary was used. The running buffer was 50 m*M* disodium hydrogenphosphate, 10 m*M* sodium tetraborate and 40 m*M* SDS, running voltage 15 kV (outlet negative). (i) Untreated fluid. (ii) After incubation with neuraminidase, note the change in t_m for peak (b) from 12.01 to 11.48 min and the development of a peak at 9.51 min due to sialic acid. Identification of peaks (b)–(d) as in Fig. 1, but peak (c) now has a longer migration time than peak (d). The peak at 7.01 min due to an aromatic amino acid is a convenient marker to monitor changes in the electroosmotic flow.

fluids to be detectable by capillary electrophoresis [16]. They arise from normal metabolic processes and are transported by serum from where they can diffuse across the synovial membrane into synovial fluid. The raw synovial fluid example used to illustrate this series of enzyme reactions (Figs. 4 and 5) contained aromatic amino acids (t_m 7.01 min), hyaluronan (t_m 13.8 min), hypoxanthine (t_m 7.8 min), xanthine (t_m 11.1 min) and uric acid (t_m 13.4 min). The peaks generated from these components form convenient markers from which to detect changes in the migration time for peak b due to the enzyme reactions, while allowing any variations due to changes in the electroosmotic flow to be detected. The migration time for hyaluronan in this sample (Fig. 4i) is longer than that for uric acid and this situation is frequently encountered with samples from patients having rheumatoid arthritis [14,15].

Neuraminidase specifically cleaves terminal N-acetylneuraminic acid residues. The electropherogram obtained after treatment of the synovial fluid sample with this enzyme is illustrated in Fig. 4ii. The peak b then appeared at shorter migration times (11.48 min) and a new peak with t_m 9.53 min appeared. The compound responsible for this new peak passed through a size exclusion filter which retained both the substance giving rise to peak b and hyaluronan. A capillary electropherogram of the filtrate is shown in Fig. 5i and this filtrate was used in further tests. The peak with t_m 9.53 min (9.45 min in Fig. 5i) was confirmed as N-acetylneuraminic acid by co-injection and by further reaction with N-acetylneuraminic acid aldolase which cleaves the molecule to pyruvic acid and N-acetyl-D-mannosamine. Capillary electrophoresis (Fig. 5ii) of the products from this second enzyme reaction showed a loss of the peak due to N-acetylneuraminic acid, but the reaction products were not detected under our

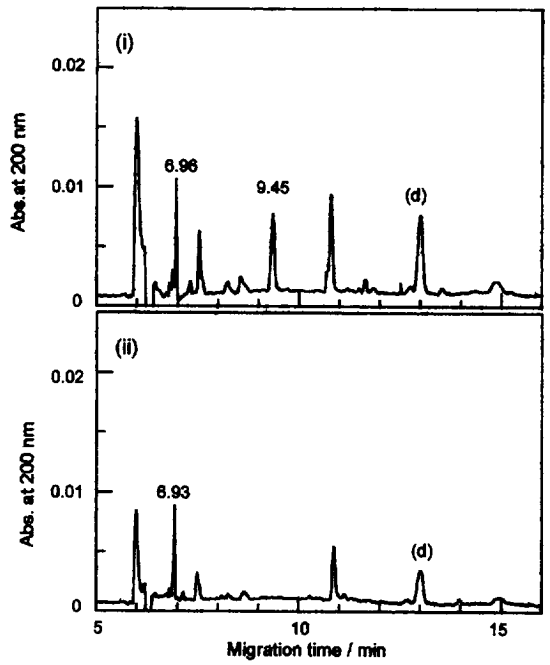

Fig. 5. Capillary electropherogram of treated synovial fluid. A 75 cm (50 cm to detector)×75 μm fused-silica capillary was used. The running buffer was 50 m*M* disodium hydrogenphosphate, 10 m*M* sodium tetraborate and 40 m*M* SDS, running voltage 15 kV (outlet negative). (i) Sample used in Fig. 4ii after passing through a size-exclusion (M_r>5000) filter. (ii) This filtrate incubated with *N*-acetylneuraminic acid aldolase. Peak (d) is due to uric acid.

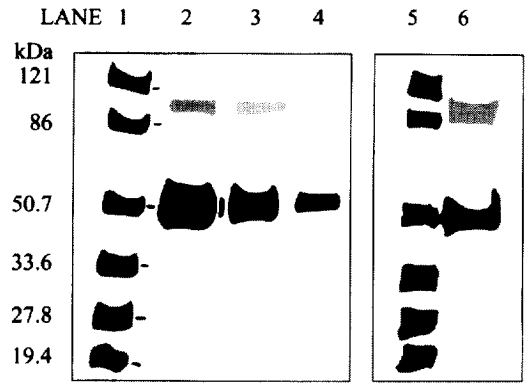

Fig. 6. Immunoblot of isolated peak (b) from human synovial fluid. AGP was separated by SDS–PAGE, blotted and probed with specific antibody followed by anti-sheep IgG/alkaline phosphatase. Lanes 1 and 5: prestained molecular mass standards; Lanes 2–4: purchased AGP (1 μg, 0.1 μg and 0.01 μg respectively); Lane 6: material of peak (b) isolated by micropreparative capillary electrophoresis.

conditions. This series of reactions indicates that the substance responsible for peak b possesses a number of N-acetylneuraminic acid units. When these units are cleaved the residue has a smaller negative charge density and therefore the observed migration time on capillary electrophoresis decreases. This suggests that peak b is due to a highly glycosylated protein such as AGP.

Isolation of the peak b fraction from synovial fluid was accomplished by repeated injection and capillary electrophoresis in a collection mode. A major problem is that the migration times of peaks gradually lengthen because the capillary walls become coated with proteins, which results in a decrease of the rate of electroosmotic flow as the runs proceed [17,18]. Preliminary trials were necessary in order to devise an automatic timing sequence for control of the separation and collection phases. The collected material was analysed by SDS–polyacrylamide gel eleactrophoresis (PAGE) followed by immunoblotting (Fig. 6), and for comparison a sample of commercial human AGP was analysed in the same way. The separated proteins were transferred to a nitro-cellulose membrane and probed using a specific AGP antibody system. The peak b sample from synovial fluid showed a positive reaction with AGP antibody and a M_r value of ca. $48 \cdot 10^3$, like that for commercial AGP on this gel.

In Fig. 4i, the peak b has a faster moving shoulder and in previous publications [13,14] we have referred to this shoulder as peak a. This shoulder is present in the capillary electropherograms of some of the samples of fluid. It is likely that glycoforms of AGP are responsible for the conglomerate of peaks a and b. Enzymatic removal of N-acetylneuraminic acid units from AGP causes a shift to shorter migration time for peak b due to the overall decrease in negative charge (compare Fig. 4i and Fig. 4ii). Peak a is likely to be due to AGP glycoforms having fewer terminal N-acetylneuraminic acid residues.

4. Conclusions

The material responsible for peak b in the capillary electropherogram of human synovial fluid is α_1-acid glycoprotein. When the N-acetylneuraminic

acid residues are cleaved enzymatically, the migration time of the core residue shortens. Examination of a range of samples of human synovial fluid has shown a fast moving shoulder on the major AGP peak and this shoulder we labelled peak a. These shoulders are caused by AGP molecules with fewer terminal N-acetylneuraminic acid residues.

Under the conditions of capillary electrophoresis used here, the major hydrophilic polymers in synovial fluid, AGP and hyaluronan, show migration times in the range of 10–16 min. AGP shows a shorter migration time than hyaluronan (peak c) This relative movement is in accord with expectation since hyaluronan, with a regular structure having one carboxylate group per two hexose units, has a higher negative charge density than AGP and is more strongly repelled by the cathode.

Acknowledgments

A CAST award (to A.R.) from the Department of Education for Northern Ireland is gratefully acknowledged. We thank Mr. R.A.B. Mollan and Dr. A.L. Bell, Musgrave Park Hospital Belfast, for provision of synovial fluid samples.

References

[1] K. Schmid, H. Kaufmann, S. Isemura, F. Bauer, J. Emura, T. Motoyama, M. Ishiguro and S. Nanno, Biochemistry, 12 (1973) 2711–2724.
[2] K. Schmid, R.B. Nimberg, A. Kimura, H. Yamaguchi and J.P. Binette, Biochim. Biophys. Acta, 492 (1977) 291–302.
[3] B. Fournet, J. Montreuil, G. Strecker, L. Dorland, J. Haverkamo, J.F.G. Vliegenthart, J.P. Binette and K. Schmid, Biochemistry, 17 (1978) 5206–5214.
[4] K. Schmid, J.P. Binette, L. Dorland, J.F.G. Vliegenthart, B. Fournet and J. Montreuil, Biochim. Biophys. Acta, 581 (1979) 356–359.
[5] H. Yoshima, A. Matsumoto, T. Mizuochi, T. Kawasaki and A. Kobata, J. Biol. Chem., 256 (1981) 8476–8484.
[6] A. Mackiewicz, R. Marcinkowska-Pieta, S. Ballou, S. Mackiewicz and I. Kushner, Arthritis Rheum., 30 (1987) 513–517.
[7] T. Pawlowski, S.H. Mackiewicz and A. Mackiewicz, Arthritis Rheum., 32 (1989) 347–351.
[8] E.C.S. van der Linden, T.W. de Graaf, M.G. Anbergen, R.M. Dekker, E.C.R. van Ommen, D.H. van den Eijnden and W. van Dijk, Glycosylation Disease, 1 (1994) 45–52.
[9] J.K. Lacki, K. Klama, W. Samborski, S.H. Mackiewicz and W. Muller, Clin. Rheumatol., 13 (1994) 598–604.
[10] K.D. Smith, M.A. Elliot, H.G. Elliot, C.M. McLaughlin, P. Wightman and G.C. Wood, J. Chromatogr. B, 661 (1994) 7–14.
[11] A.R. Poole, J. Witter, N. Roberts, F. Piccolo, R. Brandt, J. Paquin and M. Baron, Arthritis Rheum., 33 (1990) 790–799.
[12] H.B. Berntzen, Ü. Ölmez, M.K. Fagerhol and E. Munthe, Scand. J. Rheumatol., 20 (1991) 74–82.
[13] J.M. Duffy, J. Grimshaw, A. Kane, R.A.B. Mollan, P.L. Spedding and J. Trocha-Grimshaw, Anal. Proc., 31 (1994) 257–259.
[14] J. Grimshaw, J. Trocha-Grimshaw, W. Fisher, A. Kane, P. McCarron, A. Rice, P.L. Spedding, J. Duffy and R.A.B. Mollan, J. Cap. Electrophoresis, 2 (1995) 40–45.
[15] J. Grimshaw, J. Trocha-Grimshaw, W. Fisher, A. Rice, S. Smith, P. Spedding, J. Duffy and R. Mollan, Electrophoresis, 17 (1996) 396–400.
[16] K. Schmutz and W. Thormann, Electrophoresis, 15 (1994) 51–61; C.J. Petucci, H.L. Kantes, T.G. Strein and T.G. Veening, J. Chromatogr. B, 668 (1995), 241–251.
[17] K.D. Altria and Y.K. Dave, J. Chromatogr., 633 (1993) 221–225.
[18] J. Grimshaw, A. Rice and J. Trocha-Grimshaw, J. Cap. Electrophoresis, in press.
[19] U.K. Laemmli, Nature, 227 (1970) 680–685.