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ANALYSIS OF HUMAN TEAR PROTEINS BY DIFFERENT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUES

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SUMMARY

A comparison of the efficiencies of hydrophobic interaction chromatography, ion-exchange chromatography, reversed-phase chromatography and gel permeation chromatography in the separation of tear proteins was made using a variety of different buffers. Separation of immunoglobulins, lactoferrin, albumin, PMFA (protein migrating faster than albumin) and lysozyme was accomplished by gel permeation chromatography in less than 30 min using a TSK-type SW3000 column equilibrated with ammonium acetate buffer (pH 4.1) with a high reproducibility When gel permeation chromatography was used as a completely automated diagnostic method, only minute volumes (1.0 μl) of tear samples were necessary for the quantitative analysis of proteins. The other three methods proved to be more suitable for the preparation of individual tear proteins but were less suitable for their quantitation.

INTRODUCTION

The total protein concentration in human tears from healthy donors is in the range 6–10 mg/ml [1]. It has been shown that tears contain up to 60 different proteins [2], some of which are tear-specific, whereas others are identical with serum proteins. The relative molecular masses (M_r) of the individual tear proteins range from 11 800 (β-2-microglobulin) to 900 000 [immunoglob-

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ulin M (IgM)]. The four major proteins, which account for ca. 80% of the total tear protein concentration, are lactoferrin (M_r=75 000), albumin (M_r=62 000), PMFA (protein migrating faster than albumin, M_r=18 000) and lysozyme (M_r=15 000).

The analysis of tear proteins is of special interest in clinical research for the diagnosis of the pathogenesis of conjunctivitis, keratopathy, sicca syndrome, contact lens incompatibility and inborn errors of metabolism, e.g. Tay-Sachs disease [3]. Several methods for qualitative and quantitative assay of proteins in tears have been reported, including cellulose acetate gel electrophoresis, one- and two-dimensional polyacrylamide gel electrophoresis (PAGE), isoelectric focusing, crossed immunoelectrophoresis and enzyme-linked immunosorbent assay [2–5]. Recently gel permeation chromatography (GPC) by high-performance liquid chromatography (HPLC) was reported to be suitable for analysis of tear proteins [6,7].

The aim of our study was to investigate the separation efficiency of various HPLC methods to make available a fast and reliable procedure that allows a quantitative determination of the four major proteins in small volumes of tears. In addition, HPLC procedures were investigated for their ability to purify specific major and minor tear proteins.

EXPERIMENTAL

Reagents and materials

All organic solvents used as mobile phases were of HPLC grade from Rathburn (Walkerburn, U.K.), trifluoroacetic acid (TFA) was from Sigma (St. Louis, MO, U.S.A.) and HPLC-grade ammonium sulphate was from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals were of analytical-reagent grade from Merck (Darmstadt, F.R.G.).

Deionized water, bidistilled in a quartz system, was used for all aqueous solutions. All eluents were continuously degassed with helium.

Apparatus

The HPLC system from LKB (Bromma, Sweden) consisted of a 2152 HPLC controller with two 2150 pumps, a 2151 variable-wavelength monitor, a Superrac 2211 fraction collector and a Rheodyne (Cotati, CA, U.S.A.) 7125 injection valve.

The columns used were: Bio-Gel TSK DEAE-5-PW (75 mm×7.5 mm I.D.) and TSK Phenyl-5-PW (75 mm×7.5 mm I.D.) from Bio-Rad, TSK 2000SW (300 mm×7.5 mm I.D.), TSK 3000SW (300 mm×7.5 mm I.D.), TSK 3000SW (600 mm×7.5 mm I.D.) and TSK 4000SW (300 mm×7.5 mm I.D.) from Pharmacia-LKB.

For reversed-phase HPLC, columns (30 mm×4.0 mm I.D. or 250 mm×4.0

mm I.D.) packed with Nucleosil 300-7/C_4 from Macherey & Nagel (Düren, F.R.G.) were used.

Sample preparation

Since it is known that the sampling method can influence the tear protein composition [7], tears were collected at a flow-rate of 10–40 μl/min from volunteers by placing a glass capillary tube in the lower fornix of the conjunctiva. The tear samples were centrifuged for 5 min to remove any particulate matter. Samples were kept at −80°C until the analysis.

Hydrophobic interaction chromatography (HIC)

Proteins were eluted at 35°C using a 60-min linear gradient from 100% A to 100% B (buffer A, 100 m*M* sodium phosphate, pH 7.0, containing 1.7 *M* ammonium sulfate; buffer B, 100 m*M* sodium phosphate, pH 7.0). The flow-rate was 1.0 ml/min and the detection wavelength was 280 nm.

Ion-exchange chromatography (IEC)

Proteins were eluted at 35°C using the following eluents: buffer A, 10 m*M* sodium phosphate (pH 6.5); buffer B, 10 m*M* sodium phosphate (pH 6.5) containing 0.6 *M* sodium chloride. The elution conditions were as follows: 0% B for 10 min, 0 to 10% B in 20 min, 10 to 25% B in 50 min and 25 to 100% in 15 min. The flow-rate was 1.0 ml/min and the detection wavelength was 280 nm.

Reversed-phase chromatography (RPC)

Proteins were chromatographed at 35°C and a flow-rate of 0.25 ml/min. Gradient elution was started at 90% A and 10% B (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in 2-propanol or acetonitrile, pH 2.0). The proportion of solvent B (TFA in 2-propanol) was increased from 10 to 20% in 20 min, from 20 to 40% in 150 min and from 40 to 80% in 50 min. When TFA in acetonitrile was used as solvent B, the concentration of B was increased from 10 to 30% in 30 min, from 30 to 45% B in 140 min and from 45 to 80% B in 30 min.

RPC, HIC and IEC columns were returned to initial conditions by a 15-min linear gradient (0 to 80% solvent B for RPC, 0 to 100% buffer B for IEC and HIC) at a flow-rate of 1.0 ml/min and equilibrated for a further 15 min at least with solvent A or buffer A at a flow-rate of 1.0 ml/min.

Gel permeation chromatography (GPC)

GPC was carried out at room temperature at flow-rates varying between 0.5 and 1.0 ml/min, and the injection volume was up to 50 μl of tears for preparative and ca. 1.0 μl for analytical runs. The detection wavelength was 230 nm.

The eluent, 0.1 *M* ammonium acetate buffer (pH 4.1), was made from ammonia and acetic acid.

Sodium dodecylsulphate (SDS)-PAGE

Protein fractions eluted from the HPLC columns were collected, precipitated with 5% TCA (3 h, 4°C) and centrifuged for 30 min at 15 000 *g* (HIC and IEC), or concentrated in a Speed Vac concentrator (RPC and GPC). Electrophoresis was performed in 15% SDS-polyacrylamide gels under non-reducing conditions [8]. The four major human tear proteins were used as molecular mass standards. Proteins were stained using Coomassie Brilliant Blue R-250 or detected by a silver staining procedure [8].

RESULTS

Hydrophobic interaction chromatography

When tear proteins were separated by HIC, lysozyme and albumin were eluted in one peak, which was well separated from a second peak that contained PMFA and lactoferrin (not shown).

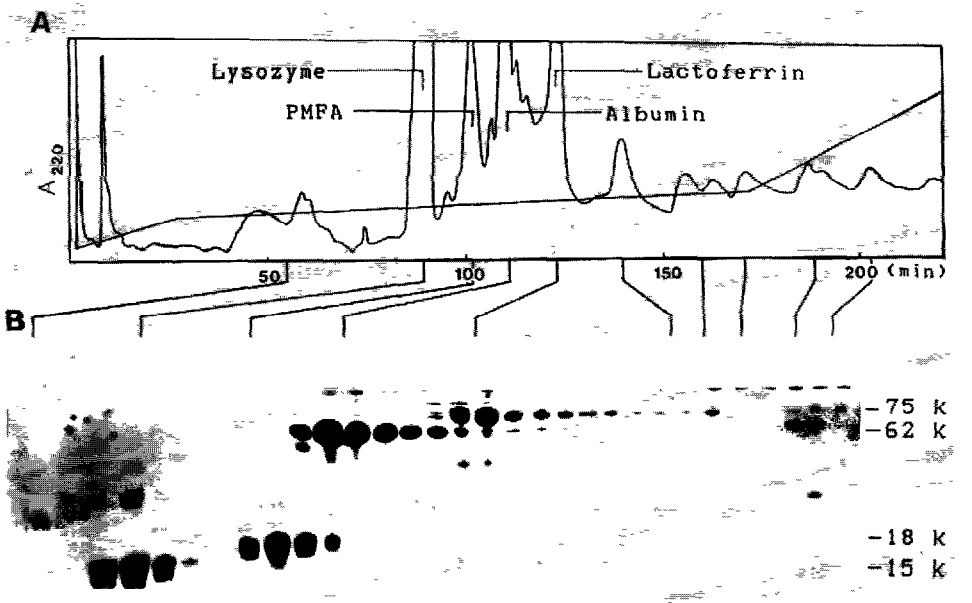

Fig. 1. Separation of tear proteins by RP-HPLC using 2-propanol as organic modifier. Sample, 20 µl of human tears; column, Nucleosil 300-7/C_4 (30 mm×4.0 mm I.D.); absorbance range, 0.16. Gradient was 10–20% B in 20 min, 20–40% B in 150 min and 40–80% B in 50 min at a flow-rate of 0.25 ml/min. (A) Elution profile; (B) the proteins contained in each fraction as analysed by SDS-PAGE.

Ion-exchange chromatography

The separation efficiency on a TSK DEAE-5-PW column at pH 8.0 was insufficient; however, at pH 6.5, lysozyme and lactoferrin were eluted close to each other and were well separated from a peak containing albumin and PMFA (not shown). A minor protein with an approximate M_r of 15 000 was eluted from the column between these two main peaks; the nature of this protein is not known.

These two methods (HIC and IEC), especially when used in combination, are very useful for the purification of specific major and minor tear proteins.

Reversed-phase chromatography

With 2-propanol as an organic modifier, the four major tear proteins were well separated from each other (Fig. 1A). A number of minor proteins were also separated and a protein with an approximate M_r of 25 000, whose identity is not known, eluted in a pure state at a retention time of 54 min (Fig. 1A and B). The resolution of albumin and PMFA was increased when short guard columns (30 mm×4.0 mm I.D.) were used instead of the usual 250 mm×4.0 mm I.D. columns, but the applied volume of tears had to be kept below 50 µl. Analysis of the individual fractions by SDS-PAGE showed that each of the major tear proteins and some of the minor proteins were present in a pure state in certain fractions (Fig. 1B).

With acetonitrile as organic modifier, lactoferrin, albumin, PMFA and lysozyme were eluted as single peaks (Fig. 2). As judged by SDS-PAGE (not

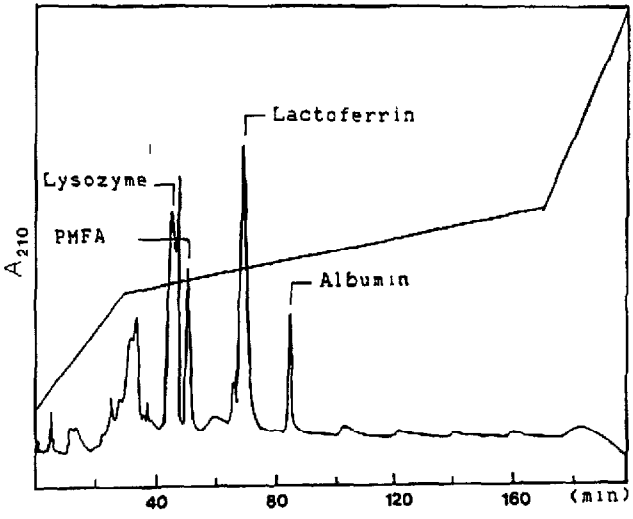

Fig. 2. Separation of tear proteins by RP-HPLC using acetonitrile as organic modifier. Sample, 20 µl of human tears; column, Nucleosil 300-7/C_4 (30 mm×4 mm I.D.); absorbance range, 0.64. Gradient was 10–30% B in 30 min, 30–45% B in 140 min and 45–80% B in 30 min at a flow-rate of 0.25 ml/min.

shown), these four major proteins and the M_r 25 000 protein (retention time 35 min, Fig. 2) were again obtained pure, whereas the other minor proteins were less well separated than with the 2-propanol system.

Gel permeation chromatography

For the separation of complex protein mixtures by GPC, sodium phosphate buffers of pH 6.5–7.5 containing 0.1–0.4 *M* salts were usually employed as eluent [9–13]. We therefore used these conditions to separate tear proteins, but with poor results (not shown). We next tested ammonium acetate buffers at different pH values (pH 4.1–7.0). At neutral pH (Fig. 3D) dimerization of albumin and lysozyme was observed. Separation efficiency was improved by lowering the pH, and the best results were achieved at pH 4.1 (Fig. 3A). Addition of sodium chloride, 100 m*M* (Fig. 3E) or 300 m*M* (Fig. 3F), did not improve the resolution. These results were obtained using a TSK 3000SW (600 mm×7.5 mm I.D.) column. In all cases, the peaks of lactoferrin and albumin overlapped. In order to improve the separation of lactoferrin and albumin, we also tested TSK 2000SW (300 mm×7.5 mm I.D.) and TSK 4000SW (300 mm×7.5 mm

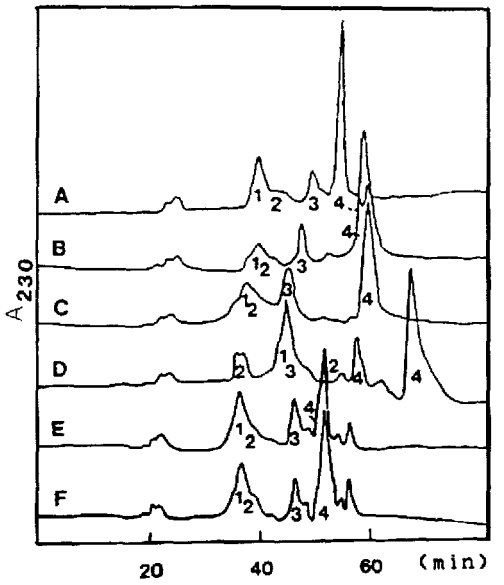

Fig. 3. Separation profiles of tear proteins by GPC on TSK 3000SW (600 mm×7.5 mm I.D.) using different buffer systems: (A) ammonium acetate (pH 4.1); (B) ammonium acetate (pH 5.2); (C) ammonium acetate (pH 6.0); (D) ammonium acetate (pH 7.0); (E) ammonium acetate (pH 4.1) containing 100 m*M* sodium chloride; (F) ammonium acetate (pH 4.1) containing 300 m*M* sodium chloride. Sample, 1 μl of human tears; absorbance range, 0.01; flow-rate, 0.5 ml/min. The proteins contained in each fraction were analysed by SDS-PAGE and the results are indicated in the figure. Peaks: 1 = lactoferrin; 2 = albumin; 3 = PMFA; 4 = lysozyme.

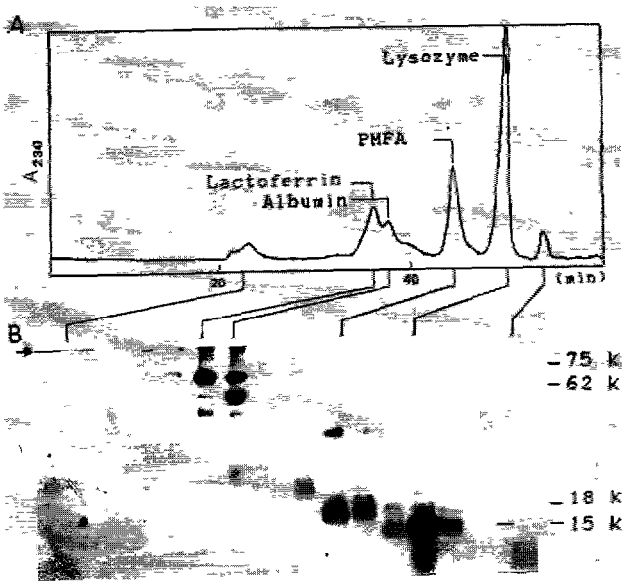

Fig. 4. Separation of tear proteins by GPC. Sample, 20 μl of human tears; column, TSK 3000SW (600 mm × 7.5 mm I.D.); absorbance range, 0.16. Ammonium acetate (pH 4.1) was used as buffer at a flow-rate of 0.5 ml/min. (A) Elution profile; (B) the proteins contained in each fraction as analysed by SDS-PAGE.

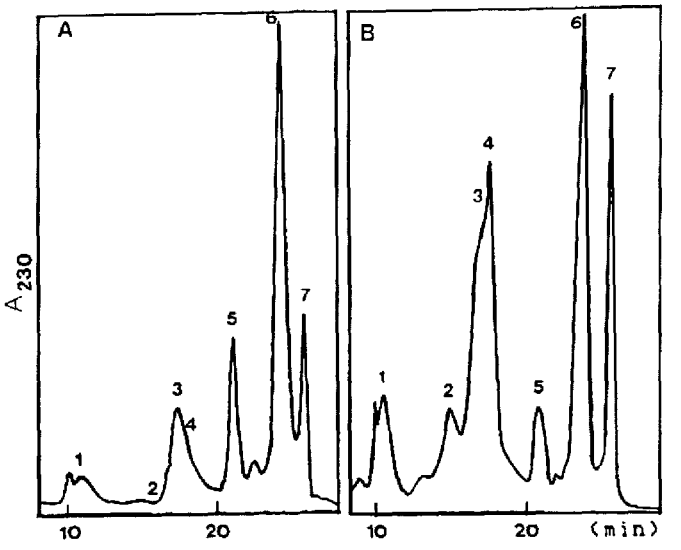

Fig. 5. Analytical separation of tear proteins by GPC Column and buffer as in Fig. 4, flow-rate, 1.0 ml/min. (A) 1 μl of tears from a healthy donor; (B) 1 μl of tears from a patient suffering from conjunctivitis. Peaks: 1 = sIgA; 2 = IgG; 3 = lactoferrin; 4 = albumin; 5 = PMFA; 6 = lysozyme; 7 = protein M_r 15 000. Lactoferrin and albumin can be distinguished by using the retention time values, which depend on albumin concentrations: low albumin concentration, 18 10–18.35 min; intermediate albumin concentration, 18.36–18.73 min; high albumin concentration, 18.74–18.92 min.

I.D.) as single columns or in combination with TSK 3000SW (600 mm×7.5 mm I.D.), but the resolution of these two proteins could not be improved.

A representative separation profile of tear proteins by GPC on a TSK 3000SW column using ammonium acetate (pH 4.1) as buffer containing no sodium chloride is shown in Fig. 4A. Under these conditions, lactoferrin, PMFA and lysozyme were obtained in a pure state, whereas albumin was contaminated with lactoferrin (Fig. 4B). Immunoglobulins can also be detected by this method. As proved by immunoblotting the faint bands (see arrow in Fig. 4B) eluted at a retention time of 22 min corresponded to secretory IgA (sIgA), whereas IgG eluted at a retention time of 33 min (see below). In normal tears only a weak signal of sIgA and no signal of IgG could be detected, according to the low immunoglobulin concentration in tears of healthy persons (compare Fig. 4 and see below).

We next attempted to adapt GPC for routine analytical purposes. By increasing the flow-rate to 1.0 ml/min and by decreasing the volume, we found that 1.0 μl of tears still generated an excellent separation profile. Fig. 5A shows the separation profile of tear proteins from a healthy donor. For comparison, the separation profile of tear proteins from a patient suffering from conjunctivitis is shown in Fig. 5B. Variations in the quantitative composition of tear proteins can be clearly seen. Owing to the increase of immunoglobulins in this sample, the peaks containing sIgA and IgG became more prominent and were clearly separated from other proteins (Fig. 5B, peaks 1 and 2). In addition, an increase in the concentration of albumin improved the separation of lactoferrin and albumin (Fig. 5B).

We have shown that tear proteins from healthy individuals and from patients with diseases of the external eye can be well separated by GPC on TSK 3000SW columns within 30 min. Since the samples may be injected in intervals of 20 min, up to three samples per hour can be analysed by this method.

DISCUSSION

A comparison of the separation efficiencies of four different HPLC procedures is given in Table I. HIC, IEC and RPC proved to be suitable for the purification of specific tear proteins but not for their quantitation. The most efficient and rapid analytical and preparative separation of tear proteins was achieved by GPC. Our results with GPC on TSK 3000SW using ammonium acetate buffer (pH 4.1) are superior to those of Boonstra and Kijlstra [14], who separated sIgA, lactoferrin and lysozyme using a Waters I-125 HPLC gel permeation column and a phosphate buffer (pH 5.28) containing 500 m*M* sodium chloride and 0.1% Tween. The GPC method described here gave a separation efficiency similar to that described by Fullard [6] who used 0.1 *M* sodium phosphate–0.5 *M* sodium chloride (pH 5.0) and a TSK 3000SW column. However, the application of GPC under the conditions described in this

TABLE I

COMPARISON OF THE DIFFERENT CHROMATOGRAPHIC TECHNIQUES USED FOR ANALYSIS OF TEAR PROTEINS

Chromatographic technique	Separation efficiency	Analysis time (min)	Non-denaturating elution conditions	Elution method
GPC	High	30–60[a]	Yes	Isocratic
IEC	Intermediate	90[b]	Yes	Gradient
HIC	Low	125[b]	Yes	Gradient
RPC	High	230–250[b]	Variable	Gradient

[a]30 min for analytical runs at a flow-rate of 1.0 ml/min; 60 min for preparative runs at a flow-rate of 0.5 ml/min.
[b]Equilibration time included.

paper allows more sensitive detection of proteins at 230 nm. Furthermore, the use of the volatile ammonium acetate buffer simplifies the preparation of proteins significantly because dialysis steps can be omitted.

In conclusion, the GPC method described appears suitable for the rapid and simple evaluation of the proteins in tears at picomole levels for clinical research and diagnostic purposes. It is an useful alternative to cellulose acetate gel electrophoresis [15,16] and SDS-PAGE [4], and is advantageous for the quantitation of immunoglobulins sIgA and IgG, PMFA and lysozyme and for an approximate quantitation of lactoferrin and albumin. The electrophoretic methods offer the advantages of a higher resolving power and the possibility to assay several samples in parallel. However, the time required for a single analysis by HPLC is much shorter. Using HPLC methods, sample application can be performed by autosampler, and the retention time and area under each peak can be determined by a computing integrator. GPC can thus be applied as a completely automated procedure for the quantitative analysis of tear proteins.

Furthermore, the chromatographic methods described here are very useful for the purification of several tear proteins in high yields. The application of HPLC thus significantly improves the preparation and analysis of tear proteins for clinical research and diagnostic purposes.

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REFERENCES

1 A.M. Gachon, J Richard and B. Dustugue, Curr. Eye Res., 2 (1983) 301.

2 A M. Gachon, P. Verrelle, G. Betail and B. Dustugue, Exp Eye Res., 29 (1979) 539.
3 N.J. Van Haeringen, Surv. Ophthalmol., 26 (1981) 84.
4 E. Mur, G Kieselbach, W. Göttinger, M. Stöffler-Meilicke and G. Stoffler, Spektrum Augenheilk , 1 (1987) 97.
5 I.A. Mackie and D.V Seal, Br. J. Ophthalmol., 68 (1984) 321.
6 R.J. Fullard, Curr. Eye Res., 7 (1988) 163.
7 A Boonstra, A.C. Breebart, C.J.J. Brinkman, L. Luyendijk, A. Kuizenga and A. Kijlstra, Curr. Eye Res., 7 (1988) 893.
8 B.D. Hames and D. Rickwood, Gel Electrophoresis of Proteins, IRL Press, Washington, DC, 1978.
9 K. Kojima, T. Manabe, T. Okuyama, T. Susuki and K. Tokunaga, J. Chromatogr., 239 (1982) 565.
10 D. Ratge and H. Wisser, J. Chromatogr., 230 (1982) 47.
11 B.B. Gupta, J. Chromatogr., 282 (1983) 463.
12 K. Fukano, K. Komiya, H. Sasaki and T Hasimoto, J. Chromatogr., 166 (1978) 47.
13 Y. Kato, K. Komiya, H. Sasaki and T. Hasimoto, J Chromatogr., 190 (1980) 297.
14 A. Boonstra and A. Kijlstra, Curr. Eye Res., 3 (1984) 1461.
15 G. Stöffler, Mol Gen. Genet., 100 (1967) 374.
16 K. Miller, W. Göttinger, Y. Deleja-Hotko, A. Mayr, M. Stöffler-Meilicke and G. Stoffler, Biol. Chem. Hoppe-Seylers, 369 (1988) 877.