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PURIFICATION OF HUMAN PLASMA LIPID TRANSFER PROTEIN USING FAST PROTEIN LIQUID CHROMATOGRAPHY

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SUMMARY

A system for the isolation of human plasma lipid transfer protein (LTP) has been devised using a combination of conventional and high-performance ion-exchange chromatography. Following initial purification by ammonium sulphate precipitation, ultracentrifugation, hydrophobic interaction and cation-exchange chromatography, appropriate fractions were further purified using the Pharmacia fast protein liquid chromatography system. Using this method of purification, human plasma LTP has been purified more rapidly and with greater recovery than with conventional column chromatography. Whereas two forms of LTP were previously reported from the authors' laboratory [LTP-I, molecular mass (M_r) 69 000 and LTP-II, M_r 55 000], with an improved chromatographic system only one form of LTP (LTP-I) has been isolated. This suggests that LTP-II may have been a fragment of LTP-I, produced during the previously used lengthy purification process.

INTRODUCTION

Plasma lipid transfer protein (LTP), which facilitates the transfer of cholesteryl ester, triacylglycerol and phospholipid between lipoproteins, has previously been purified from both human [1–5] and rabbit [6] plasma. This protein, referred to previously as LTP-I [4, 5] and cholesteryl ester transfer or exchange protein [1–3], has a molecular mass (M_r) of 63 000–69 000 [1–5] and a pI of approximately 5.0 [2, 5, 7]. Conventional methods used in the purification of the LTP involved chromatographic procedures such as hydrophobic interaction chromatography (e.g. on phenyl-Sepharose (Pharmacia Biotechnology)), ion-exchange chromatography (e.g. on carboxymethyl- or diethylaminoethyl-cellulose), lectin chromatography using concanavalin A as the functional group, hydroxyapatite chromatography, chromatofocusing and gel

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permeation chromatography. However, the procedures previously described for the isolation of LTP are long and tedious, they may not result in a high degree of protein purity, and recoveries may be poor.

Recently, Pharmacia Biotechnology have introduced the automated fast protein liquid chromatography (FPLC) system, which allows sequential computer-controlled separations on any of the above media. Pharmacia have also introduced a number of chromatographic media with the FPLC system, three of which (Mono Q and Polyanion SI, anion exchangers, and Mono S, a cation exchanger) have been used in the present study.

In this report, we present the purification to homogeneity of plasma LTP using a combination of conventional and high-performance ion-exchange chromatography. Using the FPLC system in the latter stages of the purification, we have been able to purify human plasma LTP more rapidly and with much greater recovery than with conventional column chromatography.

EXPERIMENTAL

Equipment

An automated FPLC system (Pharmacia Biotechnology, Uppsala, Sweden) was used in the latter part of the purification. The system (shown diagrammatically in Fig. 1) was controlled by an LCC-500 liquid chromatography controller (a microcomputer), programmed to perform a series of chromatographic separations in sequence. Absorbance of column eluates was monitored at 280 nm by a single-path UV-1 monitor fitted with an HR10 flow cell (10 mm path length), and recorded on a REC-482 chart recorder (0.01- 2.0 absorbance units full scale). Unwanted fractions were passed to waste, and appropriate fractions were routed via a PSV-100 solenoid valve to be stored in

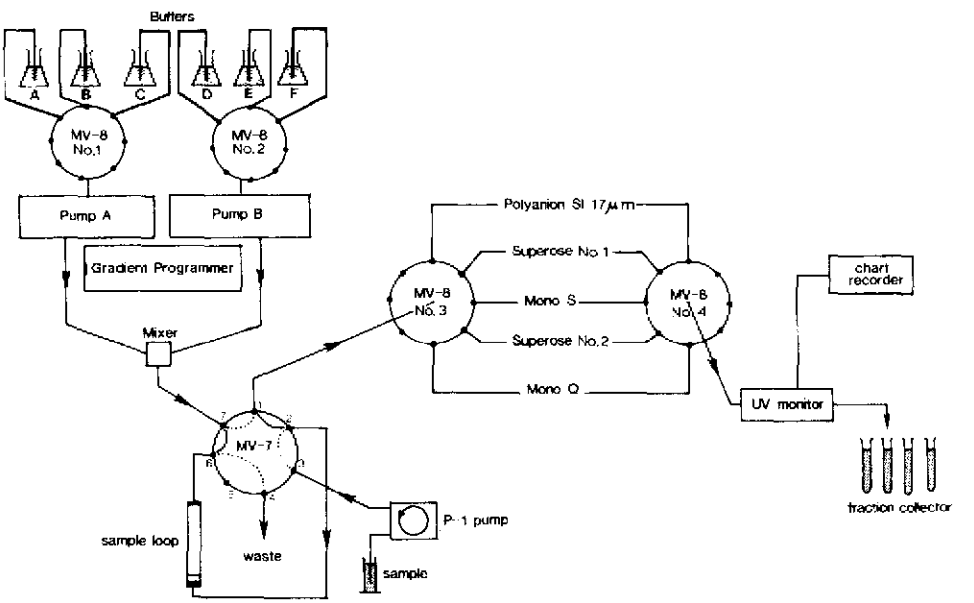

Fig. 1. Schematic representation of the FPLC system used in the purification of LTP.

the Superloop (sample loop in Fig. 1) or collected in a FRAC-100 fraction collector. Fractions containing LTP were stored in the Superloop while buffers in the pumps and lines were changed, after which the fractions were pumped on to the next chromatography column.

Anion-exchange chromatography was performed on Polyanion SI 17-μm anion exchanger packed in a 100 mm × 10 mm I.D. HR10/10 column and on a Mono Q HR5/5 column (50 mm × 5 mm I.D.). Cation-exchange chromatography was performed on a Mono S HR5/5 column (50 mm × 5 mm I.D.). Buffer exchange between ion-exchange columns was performed on one of two separate Superose 6B columns (540 mm × 16 mm I.D. and 320 mm × 16 mm I.D.).

Materials

Human plasma was obtained from the Red Cross blood bank and was stored at 4°C in the presence of 0.02% (w/v) sodium azide, 0.01% (w/v) gentamicin sulphate, 0.01% (w/v) disodium EDTA and 0.005% (w/v) chloramphenicol. The stored plasma was used within ten days. Phenyl-Sepharose CL-4B and Superose 6B, the FPLC system including Mono Q, Mono S and Polyanion SI columns, were obtained from Pharmacia Biotechnology. Carboxymethyl cellulose CM-52 was obtained from Whatmann (Kent, U.K.). [1-α,2α(n)-^{3}H]-Cholesterol, 40–60 Ci/mmol, and ACS-II scintillant were obtained from The Radiochemical Centre (Amersham, U.K.). Water was deionised and glass-distilled. All chemicals used were of analytical grade.

Buffer solutions

All buffers and samples applied to FPLC columns were filtered through a 0.22-μm sterile filter (Millipore, Bedford, MA, U.S.A.). For buffers containing urea, freshly made urea solutions were deionised with analytical-grade Amberlite MB-1 monobed resin (BDH, Poole, U.K.) until conductivity was 3 μS or less, before salts were added. All FPLC buffers contained 0.02% (w/v) sodium azide and 0.01% (w/v) disodium EDTA. Buffers for ion-exchange chromatography are given with the figure legends.

Lipid transfer protein activity assays

LTP activity was measured as the ability of protein fractions to facilitate the transfer of [^{3}H]cholesteryl ester from low-density lipoprotein (LDL) to high-density lipoprotein (HDL) during 3-h incubations at 37°C.

The assay for LTP activity was essentially as described by Pattnaik et al. [7] with the modifications described by Abbey et al. [6].

Electrophoresis

The column eluates were analysed with 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [8]. Polypeptide bands were visualized with a silver-staining method adapted from the method of Merril et al. [9] using a silver stain kit (Bio-Rad Labs., Richmond, CA, U.S.A.). The only modification was that the gel was treated with a 5 μg/ml solution of freshly made dithiothreitol instead of the oxidizer provided in the kit prior to silver nitrate impregnation [10].

Purification of human lipid transfer protein

LTP was purified from human plasma (approx. 2 l per purification). Ammonium sulphate precipitation of proteins, ultracentrifugation to remove lipoproteins, hydrophobic interaction chromatography on phenyl-Sepharose CL-4B and cation-exchange chromatography on cellulose CM-52 were carried out at 4°C as described previously [7] except that plasma proteins were precipitated with ammonium sulphate between 25 and 50% saturation. Fractions from CM-cellulose containing LTP activity were pooled and immediately dialysed at 4°C against 50 m*M* imidazole hydrochloride, pH 6.8, in 4 *M* urea.

All FPLC chromatographic steps were conducted at room temperature. The dialysed LTP sample was first applied to the Polyanion SI column and eluted with a gradient of sodium chloride (Fig. 2). LTP activity eluted in the leading edge of the absorbance peak, between sodium chloride concentrations of approximately 100 and 250 m*M* sodium chloride. These fractions were then pooled and desalted and buffer-exchanged on a Superose 6B column (540 mm × 16 mm I.D.) pre-equilibrated in 50 m*M* sodium acetate pH 4.8 at 1.0 ml/min

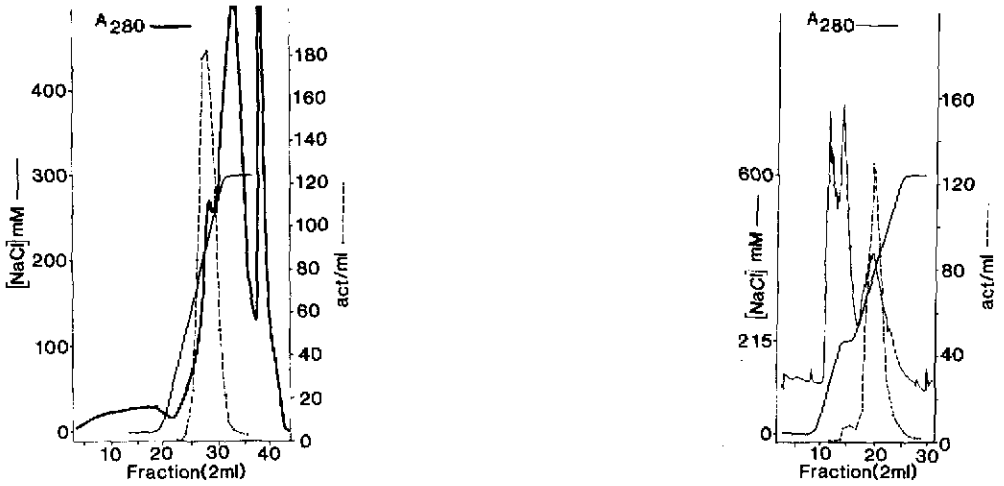

Fig. 2. Anion-exchange chromatography on Polyanion SI 17-μm anion exchanger. Fractions from CM-cellulose containing LTP activity were pooled and dialysed overnight against 50 m*M* imidazole hydrochloride pH 6.8 in 4 *M* urea, before being loaded onto the Polyanion SI column. Chromatographic conditions: buffer A, 50 m*M* imidazole hydrochloride pH 6.8 in 4 *M* urea; buffer B, buffer A + 0.5 *M* sodium chloride; flow-rate, 3.0 ml/min; sample, LTP fraction from CM-cellulose step (49.4 ml); gradient, 0—60% B in 12 min; detection, 280 nm, 0.2 a.u.f.s.

Fig. 3. Cation-exchange chromatography on Mono S HR5/5. Fractions from the Polyanion SI column containing LTP activity were pooled and loaded onto a Superose 6B column (540 × 16 mm I.D.) pre-equilibrated in 50 m*M* sodium acetate pH 4.8, at 1.0 ml/min. The protein peak from the Superose 6B column was stored on the Superloop by diverting the eluent flow via a solenoid valve. The LTP sample was then loaded onto the Mono S column pre-equilibrated with the same buffer. Chromatographic conditions: buffer A, 50 m*M* sodium acetate pH 4.8; buffer B, buffer A + 1.0 *M* sodium chloride; flow-rate, 2.0 ml/min; sample, LTP fraction from Superose 6B step (35 ml); gradient, 0—21.5% B in 5 min, 21.5% B for 2 min, 21.5—60% B in 9 min; detection, 280 nm, 0.05 a.u.f.s.

(not shown). The protein peak from the Superose 6B column was stored on the Superloop by diverting the eluent flow via a solenoid valve. The LTP sample, now in 50 m*M* sodium acetate pH 4.8, was loaded onto the Mono S column pre-equilibrated in the same buffer, and eluted with a gradient of sodium chloride (Fig. 3). The gradient was held at 215 m*M* sodium chloride for 2 min to ensure that the major contaminating protein peak was eluted before elution of the peak containing LTP. The majority of LTP activity eluted between 215 and 600 m*M* sodium chloride. The eluent from this region of the sodium chloride gradient was diverted to the Superloop, via a solenoid valve, ready to load onto the next column. The active fractions from the Mono S column were desalted and buffer-exchanged on a second Superose 6B column (320 mm × 16 mm I.D.) pre-equilibrated in 20 m*M* Tris—HCl pH 7.4 containing 4 *M* urea at 1.0 ml/min (not shown). The protein peak from the Superose 6B column was collected in the fraction collector. These fractions, containing the LTP activity, were then loaded onto the Mono Q column equilibrated in the same buffer, and eluted with a gradient of sodium chloride (Fig. 4A).

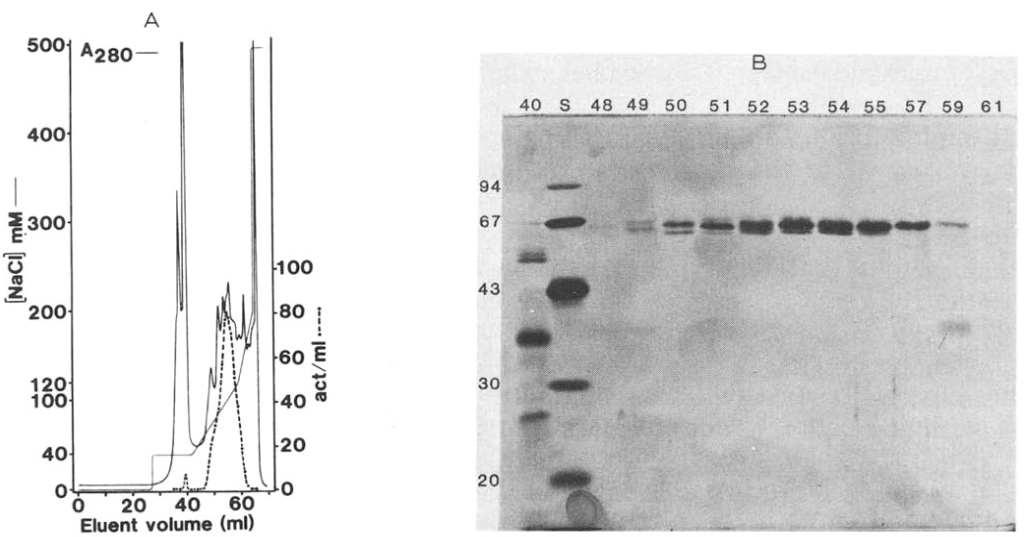

Fig. 4. (A) Anion-exchange chromatography on Mono Q HR 5/5. Fractions from the Mono S column containing LTP activity were pooled and loaded onto a Superose 6B column (320 × 16 mm I.D.) pre-equilibrated in 20 m*M* Tris—HCl pH 7.4 in 4 *M* urea, at 1.0 ml/min. The protein peak from the Superose 6B column was stored on the Superloop by diverting the eluent flow via a solenoid valve. The LTP sample was then loaded onto the Mono Q column pre-equilibrated in the same buffer. Chromatographic conditions: buffer A, 20 m*M* Tris—HCl pH 7.4 in 4 *M* urea; buffer B, buffer A + 0.5 *M* sodium chloride; flow-rate, 1.0 ml/min; sample, LTP fraction from Superose 6B step (20 ml); gradient, 8% B for 14 min, 8—24% B in 16 min, 24—40% B in 8 min, 100% B for 5 min; detection, 280 nm, 0.02 a.u.f.s. (B) Eluted fractions were analysed on SDS-PAGE (10%). Numbers above each lane correspond to eluent volume from the Mono Q column. The molecular mass of reference proteins (lane S) is given in kilodaltons.

RESULTS AND DISCUSSION

The fractionation of hydrophobic, lipid-associated proteins is not always

easy. Many of these proteins have similar properties and tend to form dimers, trimers, etc., as well as heterocomplexes. They are sometimes heterogeneously glycosylated and artefactual modification during long purification procedures may affect their structures.

Our aim was to develop a method for purification of LTP, a hydrophobic glycoprotein, using a fast and reproducible series of chromatographic steps which would increase yield and purity of the protein. The Pharmacia FPLC system has enabled us to achieve this.

The initial steps of ammonium sulphate precipitation, ultracentrifugation, phenyl-Sepharose and CM-cellulose chromatography [4, 7], were necessary to remove the bulk of protein prior to FPLC. Using a starting plasma volume of 2 l, the amount of protein was reduced to 50–100 mg after the CM-cellulose step. This amount of protein is within the loading capacity of the Polyanion SI column.

Initially, elution of LTP from each column was followed by an assay of each fraction for cholesteryl ester transfer activity. However, after several separate preparations, the chromatograms were found to be so reproducible that it was not necessary to assay at each step of the purification (small aliquots of the pooled fractions are taken at each step and assayed at the end of the run to determine recoveries). This enables us to carry out the five FPLC steps in 7–8 h.

A major contributing factor to the speed of the purification procedure has been the use of the new agarose gel matrix, Superose 6B, for desalting and

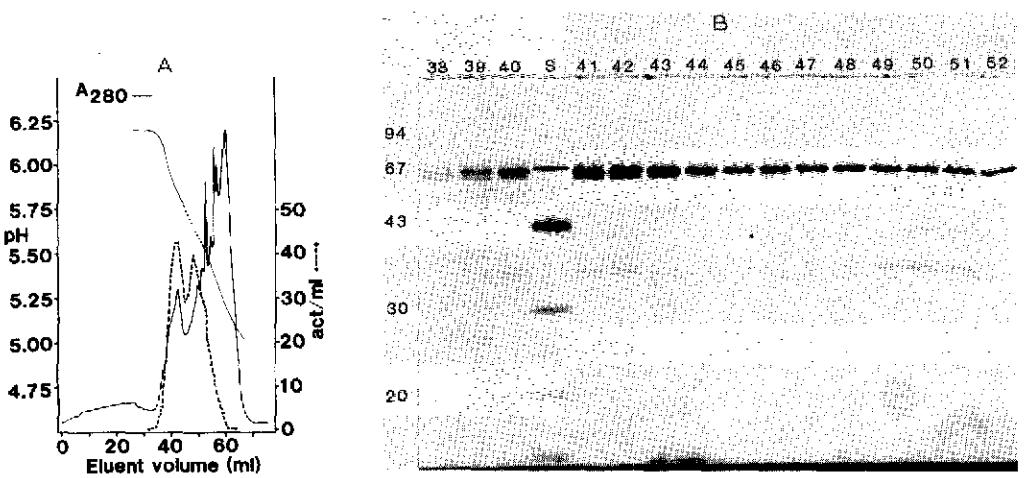

Fig. 5. (A) Anion-exchange chromatography on Mono Q HR5/5. Fractions from Mono S containing LTP activity were pooled (Fig. 4), desalted on Superose 6B (320 × 16 mm I.D.) and exchanged into 20 m*M* piperazine-hydrochloride pH 6.2 in 4 *M* urea and loaded onto the Mono Q column equilibrated with this latter buffer. Chromatographic conditions: buffer A, 20 m*M* piperazine hydrochloride pH 6.2 in 4 *M* urea; buffer B, 20 m*M* piperazine hydrochloride pH 4.5 in 4 *M* urea; flow-rate, 1.0 ml/min; sample, LTP fraction from Superose 6B step (20 ml); gradient, 0–100% B in 25 min, 100% B for 10 min; detection, 280 nm, 0.05 a.u.f.s. (B) Eluted fractions were analysed on SDS-PAGE (10%). Numbers above each lane correspond to eluent volume from the Mono Q column. The molecular mass of reference proteins (lane S) is given in kilodaltons.

buffer exchange. The highly cross-linked nature of the individual agarose bead ensures overall rigidity and, as a consequence, increased flow-rates. This has proved to be extremely useful, replacing time-consuming methods previously used such as dialysis or desalting on Sephadex G-25. For example, when desalting the pooled Polyanion SI fractions on the large Superose 6B column (up to 25 ml sample size on a 108-ml column), complete buffer exchange of the protein is achieved in 100 min with greater than 90% recovery of activity.

When examined by SDS-PAGE using the system of Laemmli [8], purified LTP from the Mono Q column migrated as two very close bands; a major band of M_r 56 000 and a minor band of M_r 63 000 (Fig. 4B). These bands could not be resolved on the phosphate buffer-based SDS-PAGE system of Weber and Osborn [11], where they appeared as a single band of M_r 69 000 (not shown). The M_r 66 000 protein has been previously referred to as LTP-I [4, 5]. In order to separate these two proteins, a buffer system employing a pH gradient was used on the Mono Q column (Fig. 5A). Separation of the two proteins was achieved yielding a pure protein of M_r 66 000 (LTP-I) (Fig. 5B, eluent volume 45—47 ml). This latter system did not afford the separation of other minor contaminants as efficiently as the Tris—NaCl gradient on Mono Q (Fig. 4A and B). We have not been able to purify the M_r 63 000 protein to determine whether it has lipid transfer activity. Its close association with LTP-I suggests that it is a glycoprotein variant of the same protein.

Data on recovery are given in Table I. It is apparent that the yield in individual steps is increased over the conventional preparation [4] in which anion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose followed by chromatofocusing and gel filtration on Sephacryl S-200 were used instead of the FPLC columns. This increase in yield can be attributed to the high resolution and speed of separation of the FPLC system.

We previously described two LTPs, LTP-I (apparent M_r 69 000) and LTP-II (apparent M_r 55 000) [4], which we had purified from human plasma by

TABLE I

PURIFICATION OF LIPID TRANSFER PROTEIN (LTP-I) FROM HUMAN PLASMA

Values given for recovery are representative of several preparations, and refer to the recovery of cholesteryl ester transfer activity (i.e. the ability of LTP-I to facilitate transfer of tracer esterified cholesterol from LDL to HDL).

FPLC			Conventional [4]		
Purification step	Recovery (%)	Recovery for each step (%)	Purification step	Recovery (%)	Recovery for each step (%)
Phenyl-Sepharose	100	—	Phenyl-Sepharose	100	—
CM-cellulose	75	75	CM-cellulose	75	75
Polyanion SI	56	75	DEAE-cellulose	37	49
Superose 6B No. 1 Mono S	45	80	Chromatofocusing	27	57
Superose 6B No. 2 Mono Q	42	93	Gel chromatography	4—5	18

classic chromatographic techniques. We noted that it was difficult to separate these proteins; indeed, only a partial separation was possible, resulting in the recovery of very small amounts of pure LTP. We suggested that the smaller lipid transfer protein might be a fragment of the larger, formed during the lengthy purification process. Since using the rapid purification system based on the FPLC, as described in this paper, we have observed LTP activity only in association with LTP-I (apparent M_r 66 000 on a Laemmli SDS-PAGE system [8], 69 000 on a Weber and Osborn gel system [11].).

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REFERENCES

1 J. Ihm, J.L. Ellsworth, B. Chataing and J.A.K. Harmony, J. Biol. Chem., 257 (1982) 4818.
2 R.E. Morton and D.B. Zilversmit, J. Lipid Res., 23 (1982) 1058.
3 A.R. Tall, E. Abbreu and J. Shuman, J. Biol. Chem., 258 (1983) 2174.
4 M. Abbey, S. Bastiras and G.D. Calvert, Biochim. Biophys. Acta, 833 (1985) 25.
5 J.J. Albers, J.H. Tollefson, C.-H. Chen and A. Steinmetz, Arteriosclerosis, 4 (1984) 49.
6 M. Abbey, G.D. Calvert and P.J. Barter, Biochim. Biophys. Acta, 793 (1984) 471.
7 N.M. Pattnaik, A. Montes, L.B. Hughes and D.B. Zilversmit, Biochim. Biophys. Acta, 530 (1978) 428.
8 U.K. Laemmli, Nature (London), 227 (1970) 680.
9 C.R. Merril, D. Goldman, S.A. Sedman and M.H. Ebert, Science, 211 (1981) 1437.
10 J.H. Morrisey, Anal. Biochem., 117 (1981) 307.
11 K. Weber and M. Osborn, J. Biol. Chem., 244 (1969) 4406.