Volume 288, number 1,2, 41–45 FEBS 10038 August 1991

ADONIS 001457939100749S

Purification and characterization of the α-tocopherol transfer protein from rat liver

Yuji Sato[1], Kenichi Hagiwara[2], Hiroyuki Arai[1] and Keizo Inoue[1]

[1] *Department of Health Chemistry* and [2] *Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113, Japan*

Received 23 May 1991

α-Tocopheral transfer protein was purified from the 10000 × *g* supernatant of rat liver. Two isoforms of the transfer protein exist, of which the isoelectric points are 5.0 and 5.1 as determined by chromatofocusing. These two isoforms have the same molecular weight; both showed molecular weight of approx. 30500 on SDS-polyacrylamide gel electrophoresis. They cannot be distinguished from each other by amino acid composition or substrate specificity.

α-Tocopherol; Vitamin E; Transfer protein

1. INTRODUCTION

α-Tocopherol, the most active form of vitamin E, is present in the membranes of intracellular organelles where it may play an important role in the suppression of lipid oxidation. The mechanism by which α-tocopherol is transported to intracellular organelles has not yet been clarified. Previous studies [1–4] showed that rat liver cytoplasm contains a protein with a molecular weight of 30 000–40 000 that binds to α-tocopherol with high affinity and specificity, although its function is not understood. Other studies suggest that the biological role of this protein is to transfer α-tocopherol between membranes. We [5] previously demonstrated that the transfer of α-tocopherol from liposomes to mitochondria was enhanced by a factor in rat liver cytosol. Murphy and Mavis [6] confirmed the presence of a factor which stimulates the transfer of vitamin E from liposomes to microsomes. Molecular properties of the protein(s) or structural relationships to other cytosolic transfer proteins have not so far been fully elucidated. The present study was undertaken to purify and characterize the protein which is responsible for α-tocopherol transfer in rat liver.

2. MATERIALS AND METHODS

2.1. *Materials*

Egg-yolk phosphatidylcholine was prepared by chromatography on neutral aluminium oxide and silicic acid. RRR-5-methyl[^{14}C]α-tocopherol [10 Ci/mol] was kindly provided by Eisai Co. (Tokyo, Japan). Glycerol tri[9, 10 (*n*)-^{3}H]oleate (0.5–1 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). Sephadex G-75 superfine, DEAE Sepharose CL-6B, PBE 94, Polybuffer 74, and Blue Sepharose CL-6B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxyapatite was purchased from Bio-Rad (Richmond, USA).

Correspondence address: Department of Health Chemistry, and Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113, Japan. Fax: 81-3-3818-3173

2.2. *Preparation of liposomes*

Liposomes composed of egg-yolk phosphatidylcholine, dicetylphosphate, butylhydroxytoluene (molar ratio 5/0.5/0.05) with a trace amount of [^{14}C]α-tocopherol (1.5×10^6 cpm) and glycerol tri[^{3}H]oleate (4.5×10^7 cpm) as a non-exchangeable marker were prepared as described previously [5].

2.3. *Preparation of crude mitochondrial fraction from rat liver*

Male Sprague-Dawley rats (350–500 g) were killed by decapitation and their livers perfused with ice-cold buffer A (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) and then rapidly excised. All subsequent operations were performed at 4°C. The livers were minced with scissors and homogenized in 2.3 vols. of buffer A. The homogenates were centrifuged at 600 × *g* for 10 min and the supernatant was centrifuged again at 10 000 × *g* for 20 min to precipitate mitochondria. The pellets (crude mitochondrial fraction) were suspended in buffer A.

2.4. *Determination of the transfer of α-tocopherol from liposomes to mitochondria*

The procedure was based on the experiments of Bloj and Zilversmit [7]. A given amount of liposomes (0.05 μmol phospholipid phosphorus) was incubated with mitochondria (0.05 mg as protein) in the presence or absence of a sample in 1 ml of buffer A. Then the mitochondria were precipitated by centrifugation at 15 000 × *g* for 15 min, and the samples of supernatant (0.8 ml) were counted. By this procedure, about 90% of liposomes were recovered in the supernatant. The percentage transfer of [^{14}C]α-tocopherol from liposomes to mitochondria was calculated from the formula:

$$\left(1 - \frac{^{14}C/^{3}H \text{ of liposome after incubation}}{^{14}C/^{3}H \text{ of liposomes before incubation}}\right) \times 100\%$$

2.5. *Purification of α-tocopherol transfer protein*

The supernatant fraction obtained after centrifugation at

15 000 × g of the liver homogenates was adjusted to pH 5.1 with 1 M HCl and allowed to stand for 30 min with stirring. The precipitated protein was removed by centrifugation at 10 000 × g for 20 min. The supernatant was adjusted to pH 7.2 with 1 M Tris, and was fractionated using ammonium sulfate. The fraction that precipitated between 30 and 60% saturation was dissolved in a small volume of buffer A and dialyzed against 20 mM Tris-HCl, 1 mM EDTA, pH 7.2.

2.5.1. *Sephadex G-75 gel filtration.* Fifteen millimeters of the sample thus obtained were applied to a Sephadex G-75 column (4.5 × 100 cm) equilibrated with 10 mM KH_2PO_4, 5 mM 2-mercaptoethanol, 10% glycerol, pH 6.8 (buffer B). The flow rate was 25 ml per hour.

2.5.2. *DEAE Sepharose CL-6B column chromatography.* The active fractions of Sephadex G-75 gel filtration were applied to DEAE Sepharose CL-6B column (1.0 × 25 cm) equilibrated with buffer B. The protein was eluted at a flow rate of 12 ml per hour with a linear gradient (2 × 150 ml) of 0-0.3 M NaCl, 10 mM KH_2PO_4, 5 mM 2-mercaptoethanol, 10% glycerol, pH 6.8.

2.5.3. *Hydroxyapatite column chromatography.* The active fractions of DEAE Sepharose CL-6B were applied to hydroxyapatite column (1.4 × 9.0 cm) equilibrated with buffer B. The protein was eluted at a flow rate of 24 ml per hour with a linear gradient (2 × 70 ml) of 10–200 mM KH_2PO_4, 5 mM 2-mercaptoethanol, 10% glycerol, pH 6.8.

2.5.4. *Chromatofocusing column chromatography.* The active fractions of hydroxyapatite were dialyzed against 25 mM histidine-HCl, 5 mM 2-mercaptoethanol, 10% glycerol, pH 6.2 (buffer C) and was applied to a column (1.0 × 25 cm) of Polybuffer 94 exchanger equilibrated with buffer C. The protein was eluted with Polybuffer 74 generating a pH gradient from 6.2 to 4.0.

2.5.5. *Blue Sepharose CL-6B column chromatography.* The active fractions of peak I on chromatofocusing were dialyzed against 5 mM KH_2PO_4, 5 mM 2-mercaptoethanol, 10% glycerol, pH 6.8 (buffer D) and applied to a Blue Sepharose CL-6B column (0.6 × 15 cm) equilibrated with buffer D. The protein was eluted at a flow rate of 24 ml per hour with a linear gradient (2 × 20 ml) of 0–1 M NaCl, 5 mM KH_2PO_4, 5 mM 2-mercaptoethanol, 10% glycerol, pH 6.8. The active fraction of peak II was dialyzed against 1 mM KH_2PO_4, 5 mM 2-mercaptoethanol, 10% glycerol, pH 6.8 (buffer E) and was applied to a Blue Sepharose CL-6B column (0.6 × 15 cm) equilibrated with buffer E. The active fraction which was obtained as a non-adsorbed fraction was applied again to a Blue Sepharose column under the same conditions as described above.

2.6. *Assay of binding of [^{14}C]α-tocopherol*

Binding of [^{14}C]α-tocopherol was determined by the method of Catignani [2]. Aliquots of the sample before gel filtration were incubated for 2 h at 25°C in the presence of [^{14}C]α-tocopherol (20 μCi/number of rats) added in 10 μl of ethanol per ml of the sample. Then the mixture was applied to Sephadex G-75 superfine and the same column operations were performed.

2.7. *Analytical procedures*

Protein was measured by the method of Lowry et al. [8]. SDS-polyacrylamide gel electrophoresis was performed on 15% slab gels as described by O'Farrell [9].

3. RESULTS

Table 1 summarizes the results of the purification of α-tocopherol transfer protein from 20 rat livers. It was previously shown by us and others that α-tocopherol transfer protein was identified in the 100 000 × g supernatant of rat liver homogenates. In our preliminary experiments, however, it was observed that pH 5.1 supernatants (see section 2) have a transfer activity approximately twice that of 100 000 × g supernatants. Therefore, we decided to utilize the present procedure as the first step of purification. As shown in Fig. 1, α-tocopherol transfer activity was dissolved after chromatofocusing into two peaks whose isoelectric points were 5.0 and 5.1, respectively. An SDS-polyacrylamide gel electrophoresis of the fractions thus obtained is shown in the insert of Fig. 1. The activity of each fraction parallels the quantity of the protein band indicated with the arrow. On the basis of these observations, we concluded that there exist two isoforms of α-tocopherol transfer protein that have the same molecular weight. The separation of these two isomers was finally achieved by Blue Sepharose column chromatography. Peak I activity was adsorbed on the column and was eluted by increasing the NaCl concentration (Fig. 2A). Peak II activity, on the other hand, was not appreciably adsorbed on the column, but the activity was retarded and eluted slightly after the bulk of non-adsorbed proteins (Fig. 2B). Overall purification from the supernatant of the acid precipitation was 1427-fold (I) and 932-fold (II) (Table I). SDS-PAGE of the fraction from the Blue Sepharose column is shown in Fig. 3A. The active fraction revealed a single major band. The molecular

Table I

Purification of α-tocopherol transfer protein from rat liver

Procedure	Total protein (mg)	Total activity (U* × 10^2)	Yield (%)	Specific activity (U*/mg)	Purification (Fold)
Acid precipitation	11718	43.8	100.0	0.37	1.0
Ammonium sulfate precipitation	3150	20.0	45.6	0.63	1.71
Sephadex G-75 superfine	127	19.1	43.6	15.0	40.5
DEAE Sepharose CL-6B	38.5	17.6	40.2	45.7	124
Hydroxyapatite	10.1	17.1	39.6	170	458
Chromatofocusing(I)	4.1	6.26	14.3	153	414
(II)	2.14	5.13	11.7	240	649
Blue Sepharose CL-6B(I)	1.0	5.28	12.0	528	1427
(II)	3.0	5.1	11.6	270	730
2nd Blue Sepharose CL-6B(II)	0.8	2.76	6.3	345	932

*1 U = 10% [^{14}C]α-tocopherol transfer/30 min

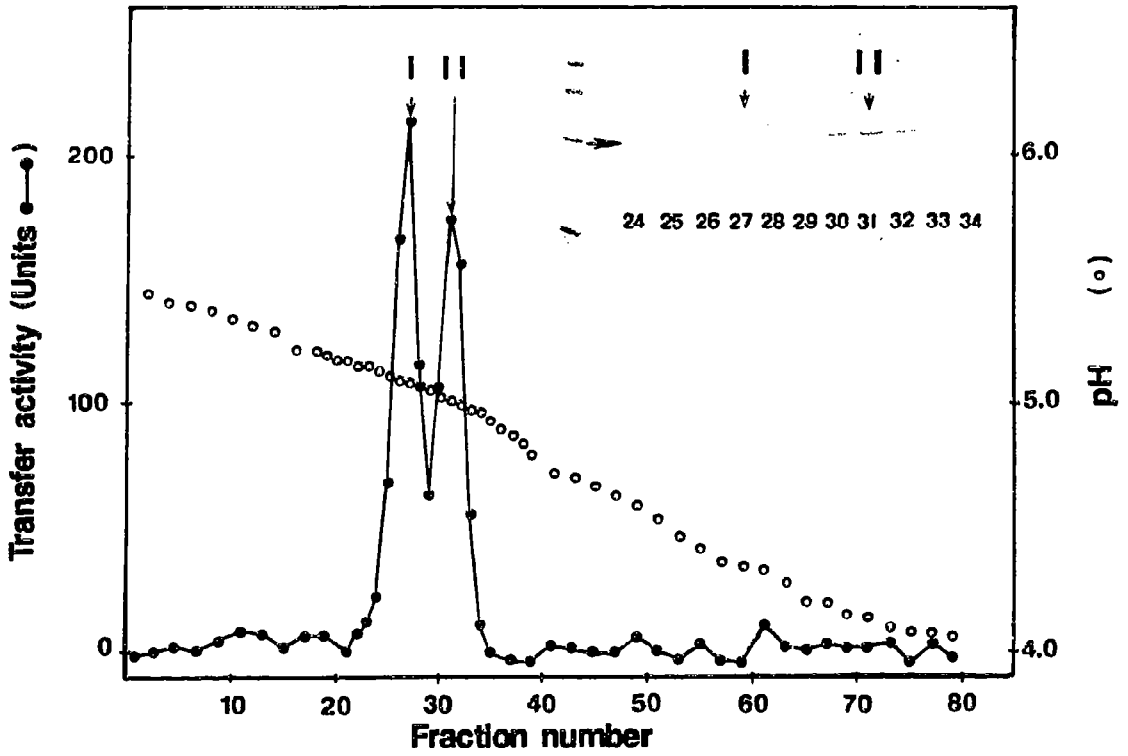


Fig. 1. Chromatofocusing of α-tocopherol transfer protein. The active fractions from a hydroxyapatite column were applied to a column (1.0×25 cm) of Polybuffer 94 exchanger previously equilibrated with buffer C. The protein was eluted with Polybuffer 74 generating a pH gradient from 6.2 to 4.0 (○), [^{14}C]α-tocopherol transfer activity of a 100 μl aliquot of each fraction; (●), pH of the fraction. The insert shows SDS-polyacrylamide gel electrophoresis of the α-tocopherol transfer protein. The number indicates the fraction number. I and II indicate the fraction of peak I and peak II, respectively.

weight of the protein calculated from this electrophoresis was 30 500 (Fig. 3B). The notion that these proteins are responsible for α-tocopherol transfer activity was further confirmed by the observation that the '30 500 protein' band exclusively co-migrated with the transfer activity throughout the purification steps after DEAE-Sepharose chromatography (data not shown).

Amino acid analysis of the purified proteins showed a similarity in the amino acid composition between these isoforms (Table II). In our preliminary observations, both isoforms are most likely to be amino-terminally blocked. The substrate specificity of the purified proteins was examined by competition studies with 500-fold excess of several unlabeled tocopherol

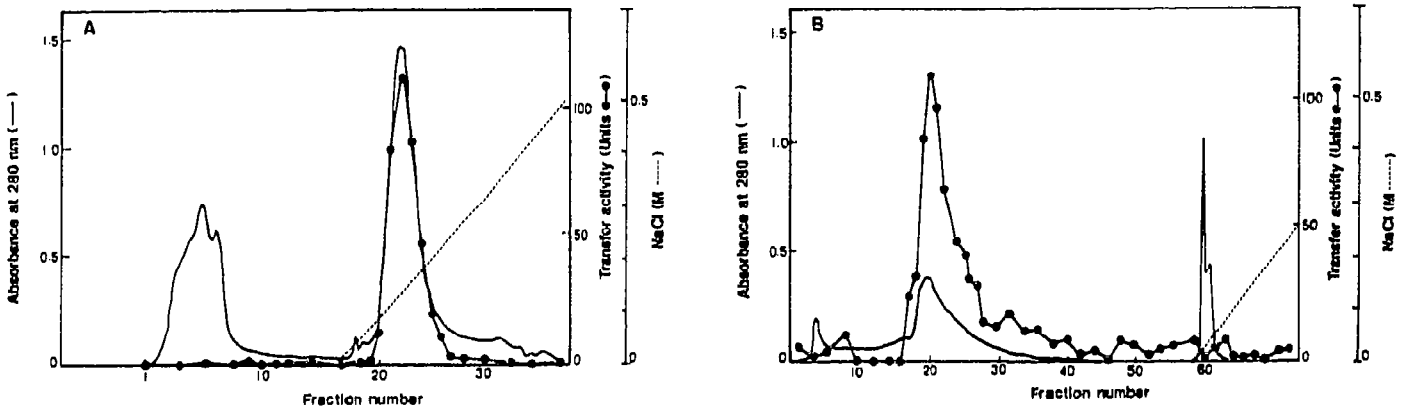
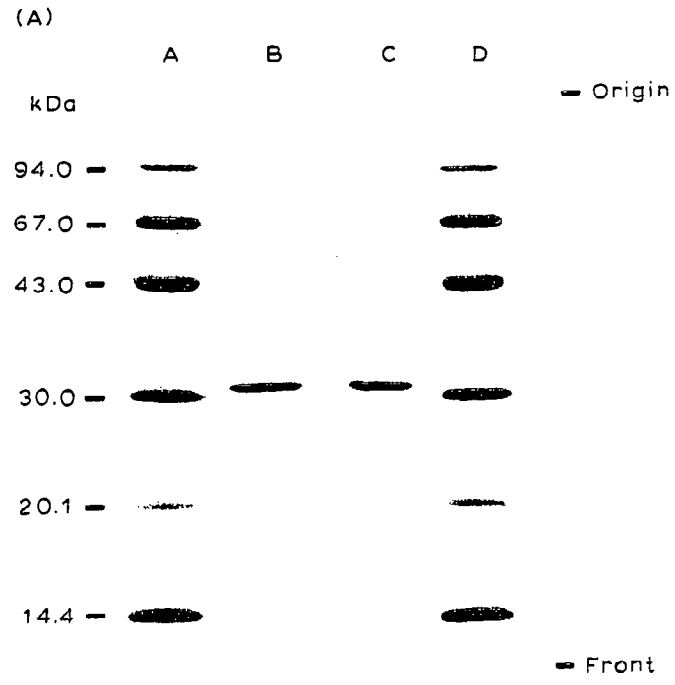


Fig. 2. Elution profile of α-tocopherol transfer proteins from a Blue Sepharose CL-6B column. Elution profiles from Blue Sepharose CL-6B column of the peak I fraction (A) and the peak II fraction (B), both of which were obtained from a chromatofocusing column. The conditions were as described in section 2. (●), [^{14}C]α-tocopherol transfer activity of a 50 μl aliquot of each fraction; (——), absorbance at 280 nm.



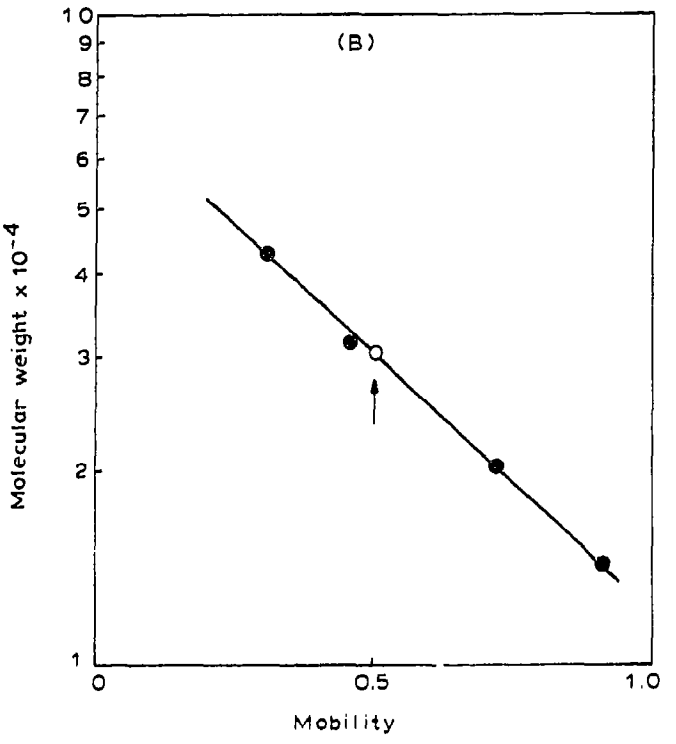


Fig. 3. SDS-polyacrylamide gel electrophoresis and molecular weight determination of the α-tocopherol transfer protein. (A) 15% polyacrylamide. Lanes A and D molecular size markers; lane B, peak I; lane C, peak II. Molecular size markers from top to bottom: phosphorylase b (94 000 Da), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), and α-lactoalbumin (14 400). Staining: Coomassie brilliant blue. (B) Molecular weight determination. The apparent molecular weight was estimated using the data shown in A.

Table II

Comparison of amino acid residues of the two isoforms of α-tocopherol transfer protein

	mol%	
Amino acid	I	II
Asp(n)	10.3	9.7
Thr	3.8	4.0
Ser	5.8	5.7
Glu(n)	12.9	9.5
Pro	6.8	6.5
Gly	7.5	8.9
Ala	6.2	6.8
Val	5.8	6.0
Met	1.9	2.2
Ile	5.4	6.7
Leu	10.9	10.7
Tyr	3.0	3.0
Phe	5.0	5.2
Lys	5.1	5.0
His	3.1	3.0
Arg	7.1	6.9

Amino acid analysis was performed following acid hydrolysis of the purified proteins (25 μg) for 24 h.

Table III

Effect of the incorporation unlabeled tocopherol derivatives into liposomes on the transfer of [^{14}C]-α-tocopherol

Competitor	[^{14}C] α-Tocopherol transfer (%/30 min)	
	I	II
None	16.1	14.6
α-Tocopherol	0.0	0.0
β-Tocopherol	0.0	0.0
γ-Tocopherol	5.8	5.3
δ-Tocopherol	11.6	10.0
α-Tocopherol acetate	13.0	12.9
α-Tocopherol quinone	16.2	13.8
Cholesterol	16.0	15.0

Liposomes composed of phosphatidylcholine, bis(hexadecyl)-phosphate and various tocopherol derivertives or cholesterol (molar ration 10 : 1 : 1, 0.2 μmol phospholipid per tube) with trace amounts of radioactive tocopherol were incubated with mitochondria at 37°C for 30 min in the presence of 1.0 μg of the purified transfer proteins.

analogues (Table III). Both isoforms showed a similar specificity: excess unlabeled α-tocopherol markedly reduced the transfer of labeled α-tocopherol. β, γ and δ-Tocopherols also decreased the transfer rate but to a lesser extent. α-Tocopherol quinone, α-tocopherol acetate or cholesterol had essentially no effect on the transfer rate. These results suggest that the purified transfer proteins recognize a certain tocopherol structure as a substrate and that the two isoforms are not distinguishable with respect to the substrate specificity.

4. DISCUSSION

We purified rat liver α-tocopherol transfer protein(s) to an electrophoretically homogeneous state and showed that there are at least two isoforms having isoelectric

points of 5.0 and 5.1, respectively. These two isoforms are not distinguishable with respect to molecular weight (30 500), amino acid composition and substrate specificity. The molecular weight of the transfer proteins calculated for the native protein on gel filtration column chromatography (see [5]) is in good agreement with the molecular weight determined by SDS-polyacrylamide gel electrophoresis in the present experiment, suggesting that the transfer protein may catalyze the α-tocopherol transfer as a monomeric form.

It was shown by several groups [1–4, 10] that rat liver cytosol has a α-tocopherol binding protein with a molecular size of approximately 30 000. We also followed α-tocopherol binding activity by labeling the pH 5.1 supernatant with [^{14}C]α-tocopherol before subsequent purification. The radioactivity was co-eluted with the transfer activity, indicating that the 30 500 proteins can bind α-tocopherol and stimulate the transfer of this vitamin between membranes.

A major challenge in the future will be to characterize the structural and functional relationships between these two isoforms.

REFERENCES

[1] Rajaram, O.V., Fatterpaker, P. and Streenivasan, A. (1973) Biochem. Biophys. Res. Commun. 52, 459–465.
[2] Catignani, G.L. (1975) Biochim. Biophys. Res. Commun. 67, 66–72.
[3] Catignani, G.L. and Bieri, J.G. (1977) Biochim. Biophys. Acta 497, 349–357.
[4] Behrens, W.A. and Madere, R. (1982) Nutr. Rep. Int. 25, 107–112.
[5] Mowri, H., Nakagawa, Y., Inoue, K. and Nojima, S. (1981) Eur. J. Biochem. 117, 537–542.
[6] Murphy, D.J. and Mavis, R.D. (1981) J. Biol. Chem. 256, 10464–10468.
[7] Bloj, R. and Zilversmit, D.B. (1977) J. Biol. Chem. 252, 1613–1619.
[8] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[9] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[10] Kaplowitz, N., Yoshida, H., Kuhlenkamp, J., Slitsky, B., Ren, I. and Stoltz, A. (1989) Ann. N.Y. Acad. Sci. 570, 85–94.