ELSEVIER

Journal of Chromatography B, 783 (2003) 191–197

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Mitochondrial creatine kinase with atypical p*I* values detected in serum of a patient with ovarian hepatoid yolk sac tumor

Fusae Kanemitsu[a,*], Takeshi Kageoka[a], Shohei Kira[b]

[a]*Clinical Laboratories, Kurashiki Central Hospital, 1-1-1 Miwa, Kurashiki 710-8602, Japan*
[b]*Department of Public Health, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*

Received 25 February 2002; received in revised form 27 August 2002; accepted 27 August 2002

Abstract

Atypical mitochondrial creatine kinase (creatine *N*-phosphotransferase, CK, EC 2.7.3.2) was detected in the serum of a patient with carcinoma of germ cell origin, probably hepatoid yolk sac tumor. The p*I* of the oligomeric atypical mitochondrial CK (Mi-CK) was found at the acidic side compared to that of the typical ubiquitous Mi-CK (uMi-CK), while the molecular size of the atypical Mi-CK was similar to that of the typical uMi-CK. The p*I*s of the oligomeric and the dimeric atypical Mi-CKs became the same as those of the typical uMi-CK upon treatment with 2-mercaptoethanol. Therefore, the atypical Mi-CK was suggested to be an oxidized form of uMi-CK, and the oxidation might have occurred in the mitochondria because the oligomeric atypical Mi-CK had atypical p*I*s. The physicochemical characteristics of the oxidized uMi-CK were similar to those of the typical uMi-CK.

Keywords: p*I* values, atypical; Enzymes; Creatine kinase

1. Introduction

Creatine kinase (creatine *N*-phosphotransferase, CK, EC 2.7.3.2) is found in the cytosol and the mitochondria of cells and catalyzes the reversible exchange of high-energy phosphoryl between ATP and phosphocreatine [1–4]. Cytosolic CK isoenzymes are dimers consisting of muscle-type (M) and/or brain-type (B) subunits. CK-MM is expressed at high levels in skeletal muscle and CK-BB is expressed in brain and smooth muscle. CK-MB, which is a hybrid of M and B subunits, is found in cardiac muscle [4]. In contrast to cytosolic CKs, mitochondrial CKs (Mi-CKs) exist as both octamers and dimers [5,6]. Mi-CKs are found exclusively in the mitochondrial compartment, attached to the outer surface of the mitochondrial inner membrane, but only the octamer can interact with both the inner and outer mitochondrial membranes [7–9].

Two genetic types of Mi-CK are known: the ubiquitous Mi-CK (uMi-CK) and the sarcomeric Mi-CK (sMi-CK) [10–12]. uMi-CK and sMi-CK are encoded by two different genes and are expressed in a tissue-specific manner; uMi-CK is expressed in brain, smooth muscle and other tissues, while sMi-CK is expressed in cardiac muscle and skeletal muscle [13,14]. The Mi-CK expressed in ovarian tissue is a ubiquitous one [15]; however, an atypical

*Corresponding author. Fax: +81-86-427-8021.

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00640-2

Mi-CK was detected in the serum of a patient with ovarian cancer (carcinoma of germ cell origin, probably hepatoid yolk sac tumor). p*I*s of the atypical Mi-CK were different from those of the typical uMi-CK but became the same as those of the typical uMi-CK upon treatment with 2-mercaptoethanol (2-ME). Therefore, in the present study, heterogeneity and enzymatic characteristics of the atypical Mi-CK are investigated.

2. Experimental

2.1. Materials

The materials employed here included the following: Autopack CK NAC, Autopack CK-MB and marker proteins for IEF and molecular masses (Roche Diagnosis, Tokyo, Japan), Sephadex G-200 superfine, agarose and carrier ampholytes for IEF (Amersham Pharmacia Biotech, Tokyo, Japan), carboxypeptidase B (Sigma, St. Louis, MO, USA), Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) and Cellogel membrane (Chemetron, Milan, Italy).

2.2. Specimens

CK isoenzymes in the sera of 187 patients with malignant tumors were separated by electrophoresis and Mi-CK was detected in the sera of 86 patients. Mi-CK isoforms in the sera of 20 of the 86 patients with more than 100 IU/l non-CK-M subunit activities were separated by IEF and the atypical Mi-CK was detected in the serum of one patient in the present study. The patient was a 62-year-old woman with advanced carcinoma of the ovary (carcinoma of germ cell origin, probably hepatoid yolk sac tumor) and serum samples obtained from the patient were used for the study. Serum from a patient with rectal cancer was used as a sample of typical uMi-CK.

2.3. Assay methods

Serum CK and non-CK-M subunit activities were determined with Autopack CK NAC and Autopack CK-MB, respectively, at 37 °C on an Hitachi 7450 analyzer. Reference levels of CK and non-CK-M subunit activities for women were 20–160 and 2.0–11.0 IU/l, respectively.

Agarose gel IEF was carried out on supporting matrices using an IEF system (Joko, Tokyo, Japan). The agarose plate used (124×80×0.5 mm) consisted of 1% agarose and 2% ampholytes (pH range 5–9). First 5-ml of serum sample was applied to the agarose plate, and 0.2 *M* citric acid and 0.5 *M* ethanolamine were used as anode and cathode solutions, respectively [13]. Focusing was carried out at 4 °C, at 300–1200 V for 120 min (power: 0.8–4.8 W). The focused Mi-CK isoforms were stained with a mixture of one pack of Autopack CK NAC, 75 mg/l nitroblue tetrazolium and 7.5 mg/l phenazine methosulfate at 37 °C for 30 min. Counter-immuno-IEF was carried out by applying 5 μl of anti-Mi-CK antiserum at a position 1.0 cm anodic to the sample application point [16]. Mi-CK was electrophoresed at 200 V per 10 cm for 17 min on a Cellogel membrane.

In order to examine the characteristics of the atypical Mi-CK, the Mi-CK was subjected to chromatography on a Sephadex G-200 superfine column and was treated separately with 2 *M* urea, 0.0–0.2% hydrogen peroxide, 0–250 m*M* 2-ME at 27 °C for 15 min, and with 900 U/l carboxypeptidase B at 37 °C for 30 min [16].

2.4. Isolation of Mi-CK and preparation of anti-Mi-CK antibody

Human cardiac muscle was homogenized in 10 m*M* Tris–HCl buffer, pH 7.4, and centrifuged at 1700 *g* for 10 min at 4 °C, and then the supernatant was further centrifuged at 12 000 *g* for 10 min [16]. The pellet was washed with Tris–HCl buffer until CK activity in the supernatant became negligible, and a mitochondrial pellet was finally obtained by centrifugation at 12 000 *g* for 10 min at 4 °C. The mitochondria were suspended in 50 m*M* phosphate buffer, pH 7.4, incubated at 4 °C for 60 min and centrifuged at 105 000 *g* for 30 min at 4 °C. The supernatant containing Mi-CK was chromatographed on a DEAE Sephacel column (0.9×15 cm) that had been equilibrated with 50 m*M* Tris–HCl buffer, pH 7.4, containing 30 m*M* NaCl, and the eluate was applied to a Sephadex G-200 superfine column. Mi-CK fractions eluted from the column were concen-

trated and approximately 5 mg of Mi-CK was emulsified with an equal volume of complete Freund's adjuvant.

First 1 ml of the emulsion was injected intramuscularly into the back of a rabbit and booster injections were given with adjuvant on days 7, 14 and 28 after the initial injection. Then 7 days after the last booster injection, the rabbit was bled from the ear vein, and the serum containing anti-human Mi-CK antibody was collected. Non-specific antibodies in the anti-serum were absorbed by incubation with normal human serum at 37 °C for 2 h, followed by incubation at 4 °C for 24 h and centrifugation. CK activity and complements in the antiserum were inactivated by heating at 56 °C for 30 min. The anti-Mi-CK antiserum was confirmed to react with only Mi-CK and not CK-MM or CK-BB by the Octerlony test [16].

3. Results

Fig. 1A shows serum CK isoenzyme patterns of the patient. A slightly broad band from the position of CK-MM to the cathodic side of CK-MM was detected by electrophoresis. The broad band migrated to the CK-MM position after treatment with 2 *M* urea. The serum CK enzymogram of the patient differed from that of normal ovarian tissue extract, and was CK-BB dominant.

Fig. 1B shows IEF patterns of serum CK of the patient. Broad CK bands were detected at p*I* 7.0–7.8 and the cathodic edge of the broad band was p*I* 0.1 away from the anodic side of the typical uMi-CK. After urea treatment, a band at p*I* 6.75 and several minor bands at the anodic side of p*I* 6.75 were detected, in addition to the typical bands of uMi-CK at p*I* 7.0 and 6.9 (Fig. 1C); however, the bands at p*I* 6.8 and 6.7 were not detected.

The serum CK of the patient reacted with anti-Mi-CK antibodies and migrated toward the anodic side (Fig. 2A); therefore, the atypical CK was considered to be Mi-CK. In order to determine the molecular size of the atypical Mi-CK, the Mi-CK was subjected to chromatography on a Sephadex G-200 superfine column. The elution patterns of the atypical Mi-CK by the column chromatography showed two CK peaks corresponding to fractions G-M and A (Fig. 2B). The atypical CK at p*I* 7.0–7.8 was detected in fraction G-M, while the atypical Mi-CK at p*I* 6.75 and the typical Mi-CK at p*I* 7.0 were detected in fraction A (Fig. 2C). Therefore, the molecular sizes

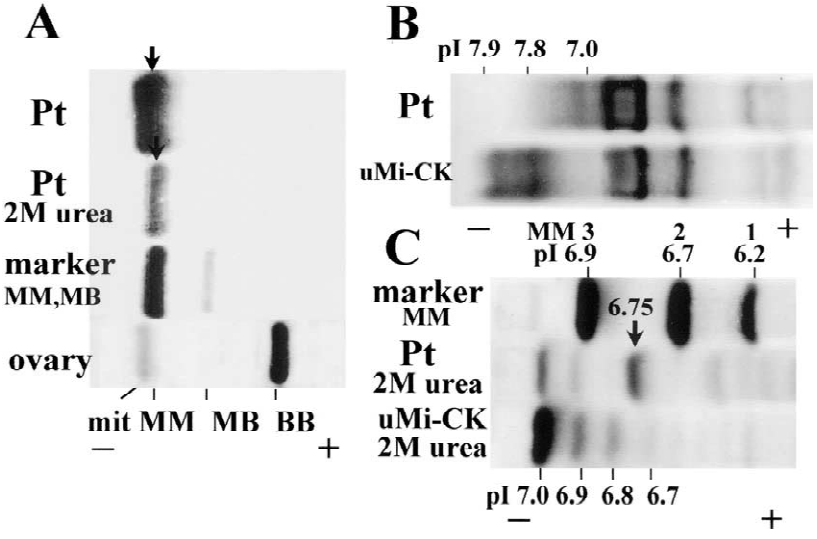

Fig. 1. (A) CK isoenzyme patterns of the sera and the tissue extract of normal ovary. Pt, patient's serum; Pt 2 *M* urea, patient's serum after treatment with 2 *M* urea at 27 °C for 15 min. (B) IEF patterns of serum CK of the patient (Pt) and typical uMi-CK (uMi-CK). (C) IEF patterns of serum CK of the patient (Pt 2 *M* urea) and typical uMi-CK (uMi-CK 2 *M* urea) after urea treatment at 27 °C for 15 min.

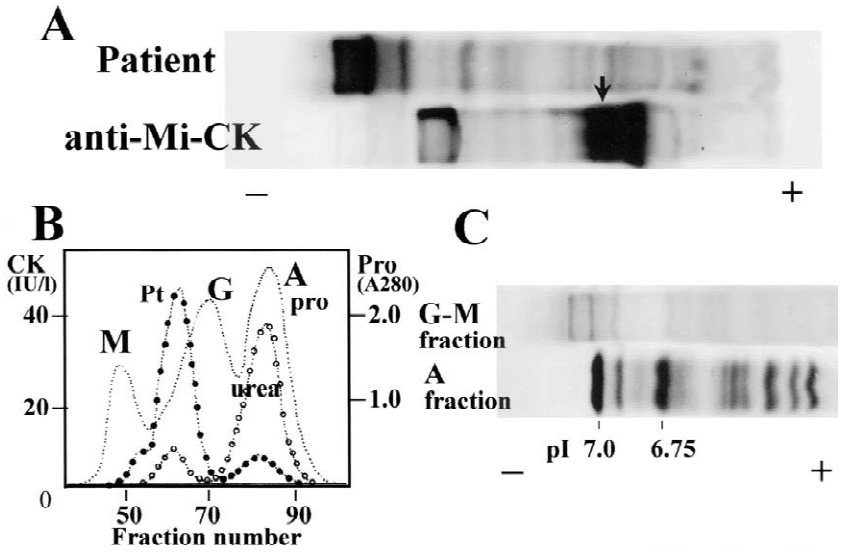

Fig. 2. (A) Reaction of the atypical CK of the patient with anti-Mi-CK antibodies. Patient, the IEF pattern of the atypical CK of the patient; anti-Mi-CK, the IEF pattern of the complexes (arrow) of the atypical CK and anti-Mi-CK antibodies. (B) Elution patterns of the atypical Mi-CK by column gel chromatography. Pt, the elution pattern of the atypical Mi-CK (· · ·●· · ·); urea, the elution pattern of the atypical Mi-CK after urea treatment (· · ·○· · ·); pro, the elution pattern of proteins in serum of the patient (· · · · · ·); A, G and M, the fractions of albumin, IgG and IgM, respectively. (C) IEF patterns of the atypical CKs in fractions G-M and A from column gel chromatography.

of the atypical Mi-CK were similar to that of the oligomeric or the dimeric form of the typical uMi-CK.

In order to examine the C-terminus of the atypical Mi-CK, the Mi-CK was treated with carboxypeptidase. After the treatment, the oligomeric atypical Mi-CK migrated toward the anodic side and was detected at the position corresponding to CK-MM3 and the cathodic side of CK-MM2 (arrows in Fig. 3A). In contrast, the oligomeric typical uMi-CK migrated toward the cathodic side of CK-MM2 and CK-MM3. The dimeric atypical Mi-CKs were detected at the anodic side of CK-MM1 after treatment with carboxypeptidase (arrows in Fig. 3B); however,

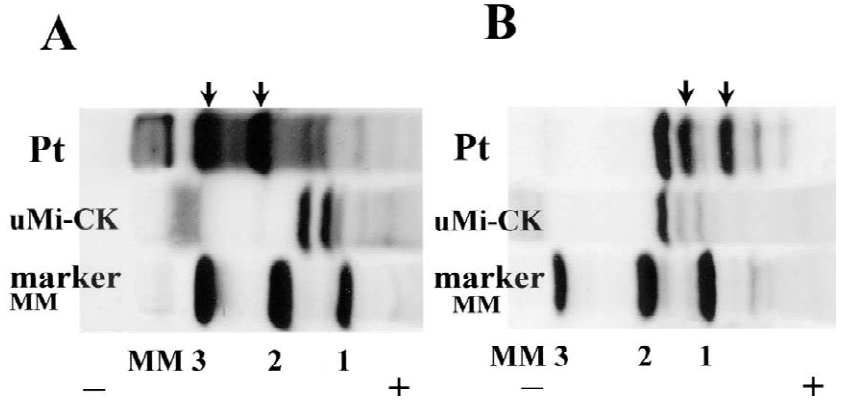

Fig. 3. (A) IEF patterns of the oligomeric Mi-CKs after treatment with 900 IU/l carboxypeptidase B at 37 °C for 15 min. Pt, the delysined atypical Mi-CK; uMi-CK, the delysined typical uMi-CK. (B) IEF patterns of the dimeric Mi-CKs after treatment with 900 IU/l carboxypeptidase B at 37 °C for 15 min. Pt, the delysined atypical Mi-CK; uMi-CK, the delysined typical uMi-CK.

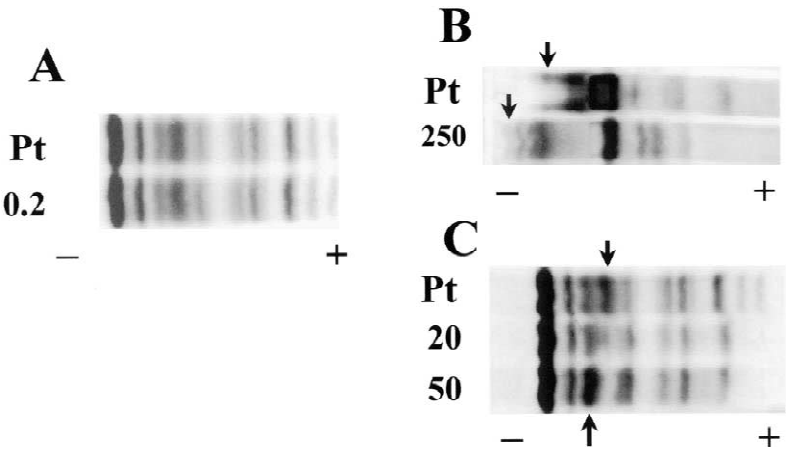

Fig. 4. (A) IEF patterns of the atypical Mi-CK after treatment with hydrogen peroxide. Pt, the atypical dimeric Mi-CK; 0.2, after treatment with 0.2% hydrogen peroxide at 27 °C for 15 min. (B) IEF patterns of the atypical oligomeric Mi-CK after treatment with 2-ME. Pt, the atypical Mi-CK; 250, after treatment with 250 m*M* 2-ME at 27 °C for 15 min. (C) IEF patterns of the atypical dimeric Mi-CK after treatment with 2-ME. Pt, the atypical Mi-CK; 20 and 50, after treatment with 20 or 50 m*M* 2-ME at 27 °C for 15 min, respectively.

dimeric typical uMi-CKs were detected between the anodic side of CK-MM2 and CK-MM1. Therefore, the p*I*s of the delysined atypical Mi-CK differed from those of the typical uMi-CK.

Typical Mi-CK is oxidized by hydrogen peroxide and its p*I*s migrate toward the anodic side; however, the p*I*s return to their original positions after treatment with 2-ME [13]. Therefore, the atypical Mi-CK was separately treated with hydrogen peroxide and 2-ME. However, after treatment with hydrogen peroxide, the IEF pattern of the atypical Mi-CK did not change (Fig. 4A). In contrast, after treatment with 2-ME, the octameric atypical Mi-CK migrated toward the cathodic side (arrows in Fig. 4B), and the dimeric atypical Mi-CK at p*I* 6.75 migrated to p*I* 6.8 (arrows in Fig. 4C).

Table 1 shows the physicochemical characteristics of the atypical Mi-CK. The characteristics were similar to those of the typical uMi-CK; therefore, the effects of oxidation on the physicochemical characteristics of the atypical Mi-CK were found to be minimal.

4. Discussion

Mi-CK levels are elevated in the sera of some cancer patients and are considered to be an advanced prognostic indicator [17,18]. In cancer patients, especially those with advanced gastric cancers, Mi-CK is detected in the sera at high rates (31–75%); however, in advanced ovarian cancer patients, Mi-CK is detected at 25% [17]. The characteristics of Mi-CK in the sera of cancer patients were similar to those of typical Mi-CK and the characteristics of atypical Mi-CK have not been reported thus far. In the present study, the atypical Mi-CK detected in the serum of the patient with ovarian yolk sac tumor had slightly higher p*I*s than the typical uMi-CK. Mi-CK

Table 1
Physicochemical characteristics of uMi-CKs (mean±SE)

	K_m (m*M*)	E_a (kJ/mol)	45 °C/20 min (%)	5 m*M* iodoacetamide (%)
Sample	3	4	2	2
Atypical uMi-CK	0.69±0.02	110.1±3.7	88.1±1.5	70.9±6.5
Typical uMi-CK	0.74±0.02	110.5±3.7	89.6±0.4	74.6±5.0

with similar p*I*s to the dimeric atypical Mi-CK was obtained by hybridization of sMi-CK and uMi-CK in vitro [16]; however, in the case investigated here, the atypical Mi-CK was not obtained by hybridization. Furthermore, the atypical part of the Mi-CK was not in the C-terminal region because the oligomeric and dimeric atypical Mi-CKs reacted with carboxypeptidase and the p*I*s of the atypical Mi-CKs after treatment with carboxypeptidase remained unchanged.

Fig. 5 shows changes of the typical uMi-CK into various forms [13] and the position of the atypical Mi-CK. The octameric uMi-CK binds to the inner and outer mitochondrial membranes [7–9], and is released into the circulation as octameric or dimeric forms [13]. The dimeric typical uMi-CK at p*I* 7.0 is a reduced form and the others are oxidized forms [13]. The reduced form is oxidized by hydrogen peroxide and migrates toward the anodic side; however, it returns to the original p*I* upon treatment with 2-ME [13]. The existence of five forms of dimeric uMi-CK consisting of three types of redox monomers is surmised (Fig. 6).

In contrast, the dimeric atypical Mi-CK in the present study was detected at p*I* 6.75 (Figs. 5 and 6). After treatment of the atypical Mi-CK with 2-ME, the octameric atypical Mi-CK migrated toward the cathodic side from the original position, and a dimeric typical uMi-CK at p*I* 6.75 migrated toward p*I* 6.8, the typical p*I* (Fig. 4B and C). From the results, the atypical Mi-CK was considered to be an oxidized form of typical uMi-CK, as shown in Fig. 6. The oligomeric atypical uMi-CK also had an atypical p*I*; therefore, the atypical uMi-CK is considered to be oxidized in the mitochondrion. The mitochondrion is the site of electron transport where remaining oxygen is converted into superoxide, and uMi-CK participates in the energy metabolism in close relation to the electron transport system [3,19,20]. Therefore, it is suggested that the patient's Mi-CK was influenced by the electron transport system.

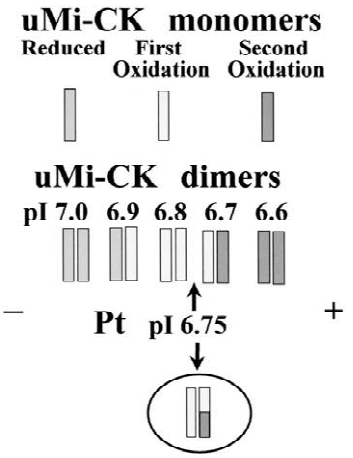

Fig. 6. Three forms of monomeric uMi-CK, five forms of dimeric uMi-CK and the atypical dimeric uMi-CK (Pt).

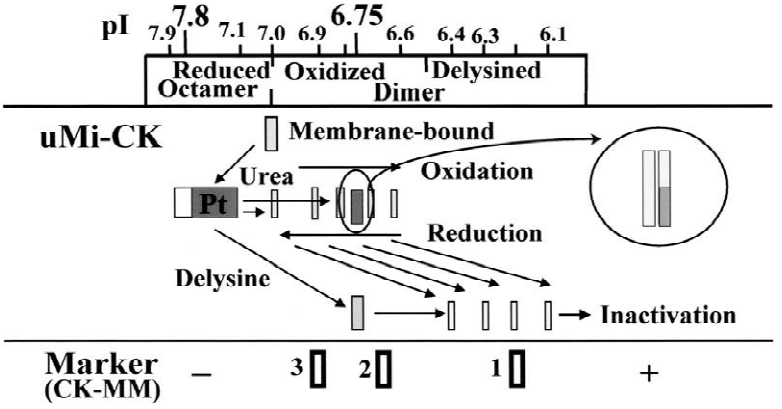

Fig. 5. Changes of the typical uMi-CK into various forms and positions of the atypical uMi-CKs.

References

[1] T. Wallimann, M. Wyss, D. Brdiczka, K. Nicolay, H.M. Eppenberger, Biochem. J. 281 (1992) 21.
[2] M. Wyss, J. Smeitink, R.A. Wevers, T. Wallimann, Biochim. Biophys. Acta 1102 (1992) 119.
[3] T. Wallimann, M. Dolder, U. Schlattner, M. Eder, T. Hornemann, E. O'Gorman, A. Ruck, D. Brdiczka, Biofactors 8 (1998) 229.
[4] P. Kaldis, T. Wallimann, Biochem. J. 388 (1995) 623.
[5] W. Kabsch, K. Fritz-Wolf, Curr. Opin. Struct. Biol. 7 (1997) 811.
[6] K. Fritz-Wolf, T. Schnyder, T. Wallimann, W. Kabsch, Nature 381 (1996) 341.
[7] O. Stachowiak, M. Dolder, T. Wallimann, Biochemstry 35 (1996) 15522.
[8] U. Schlattner, T. Wallimann, J. Biol. Chem. 275 (2000) 17314.
[9] W.R. Ellington, K. Roux, A.O. Pineda Jr., FEBS Lett. 425 (1998) 75.
[10] W. Qin, Z. Khuchua, J. Cheng, J. Boero, R.M. Payne, A.W. Strauss, Mol. Cell. Biochem. 184 (1998) 153.
[11] K. Anflous, V. Veksler, P. Mateo, F. Samson, V. Saks, R. Ventura-Clapier, Biochem. J. 322 (1997) 73.
[12] M. Eder, K. Fritz-Wolf, W. Kabsch, T. Wallimann, U. Schlattner, Proteins 39 (2000) 216.
[13] F. Kanemitsu, S. Kira, J. Chromatogr. B Biomed. Sci. Appl. 721 (1999) 171.
[14] R.C. Haas, C. Korenfeld, Z.F. Zhang, B. Perryman, D. Roman, A.W. Strauss, J. Biol. Chem. 264 (1989) 2890.
[15] R.C. Haas, A.W. Strauss, J. Biol. Chem. 265 (1990) 6921.
[16] F. Kanemitsu, J. Mizushima, T. Kageoka, T. Okigaki, K. Taketa, S. Kira, Electrophoresis 21 (2000) 266.
[17] F. Kanemitsu, I. Kawanishi, J. Mizushima, T. Okigaki, Clin. Chim. Acta 138 (1984) 175.
[18] T. Wallimann, W. Hemmer, Mol. Cell. Biochem. 133/134 (1994) 193.
[19] W.S. Kunz, Biochim. Biophys. Acta 1504 (2001) 12.
[20] J. Vina, A. Gimeno, J. Sastre, C. Desco, M. Asensi, F.V. Pallardo, A. Cuesta, J.A. Ferrero, L.S. Terada, J.E. Repine, IUBMB Life 49 (2000) 539.