ELSEVIER

Journal of Chromatography B, 728 (1999) 59–65

JOURNAL OF CHROMATOGRAPHY B

High-performance liquid chromatographic–fluorimetric assay for cathepsin A (lysosomal protective protein) activity

Toshiyuki Chikuma[a,*], Yumiko Ogura[a], Michi Kasamatsu[a], Kyoji Taguchi[b], Kenichi Mitsui[c], Takeshi Kato[d], Akira Tanaka[a]

[a]*Department of Pharmaceutical Analytical Chemistry, Showa College of Pharmaceutical Sciences, 3-3165 Higashi-tamagawagakuen, Machida-shi, Tokyo 194, Japan*

[b]*Department of Pharmaco-Therapeutics, Showa College of Pharmaceutical Sciences, 3-3165 Higashi-tamagawagakuen, Machida-shi, Tokyo 194, Japan*

[c]*Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Toin University of Yokohama, 1614 Kurogane-cho, Aoba-Ku, Yokohama 225, Japan*

[d]*Laboratory of Molecular Recognition, Graduate School of Integrated Science, Yokohama City University, 22-2 Seto, Kanazawa-Ku, Yokohama 236, Japan*

Received 3 September 1998; received in revised form 28 December 1998; accepted 6 January 1999

Abstract

A rapid and sensitive assay for the determination of cathepsin A activity is reported. This method is based on fluorimetric detection of a dansylated peptide, 5-dimethylaminonaphthalene-1-sulfonyl-L-Phe, enzymatically formed from the substrate 5-dimethylaminonaphthalene-1-sulfonyl-L-Phe-L-Leu, after separation by high-performance liquid chromatography using a C_{18} reversed-phase column and isocratic elution. This method is sensitive enough to measure 5-dimethylaminonaphthalene-1-sulfonyl-L-Phe at concentrations as low as 300 fmol, yields highly reproducible results and requires less than 7.0 min per sample for separation and quantitation. The optimum pH for cathepsin A activity was 4.5–5.0. The K_m and V_{max} values were respectively 14.9 μM and 27.91 pmol/μg/h with the use of enzyme extract obtained from mouse kidney. Cathepsin A activity was strongly inhibited by Ag^+, Hg^{2+}, diisopropylfluorophosphate and *p*-chloromercuriphenylsulphonic acid. Among the organs examined in a mouse, the highest specific activity of the enzyme was found in kidney. The sensitivity and selectivity of this method will aid in efforts to examine the physiological role of this peptidase.

Keywords: Cathepsin A; Enzymes

1. Introduction

Mammalian cathepsin A (EC 3.4.16.5, also known as 'lysosomal carboxypeptidase A' and 'lysosomal protective protein') was originally defined by Tallan et al. as the enzyme which hydrolyzes Z-Glu-Tyr at acidic pH [1]. The enzyme also demonstrates esterase and deamidase activities at neutral pH [2]. Since cathepsin A is able to hydrolyze in vitro a wide spectrum of both synthetic and bioactive peptide hormones such as Z-Phe-Leu, angiotensin II, substance P and endothelin I [2–7], it has been suggested that cathepsin A may be implicated in the in

*Corresponding author. Tel.: +81-427-211567; fax: +81-427-211588.

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00029-8

vivo metabolism of peptide hormones, although the physiological substrates of cathepsin A are still unknown. Furthermore, cathepsin A has been studied by some workers with the aim of elucidating the organ's specific physiological functions of the enzyme in the liver [8], brain [9] and kidney [10].

Lysosomal protective protein was initially identified as a glycoprotein regulating the expression of β-galactosidase (EC 3.2.1.23) and neuraminidase (EC 3.2.1.18) through the formation of a high molecular mass multienzymic complex in lysosomes [11–14]. It is synthesized in human fibroblasts as a single precursor and then processed to a mature form, consisting of 32- and 20-kDa peptides linked by disulfide bonds [11]. A deficiency of this protein in humans leads to a genetically determined metabolic storage disorder, galactosialidosis, that can be fatal to infants [15,16].

The most commonly used assay system for detecting and estimating cathepsin A activity is based on the ability of the enzyme preparation to convert the synthetic dipeptide such as Z-Phe-Leu to the corresponding Z-Phe and Leu. The degree of conversion to product is determined by derivatization of Leu formed enzymatically with ninhydrin [3,5], L-amino acid oxydase-peroxydase-*o*-dianisidine [17] and *o*-phthalaldehyde reagent [18,19]. The progress of the reaction was also monitored by measurement of the Z-Phe formed by reversed-phase high-performance liquid chromatography (HPLC) with a spectrophotometric detection [10]. A few other assays of cathepsin A activity have been reported. These include a continuous spectrophotometric assay using furylacryloyl-Phe-X dipeptides as substrates [2,20] and a liquid–liquid partition of product and substrate under the acidic pH using 5-dimethylaminonaphthalene-1-sulfonyl-L-Ala-L-Arg (N-DNS-Ala-Arg) or 5-dimethylaminonaphthalene-1-sulfonyl-L-Phe-L-Leu-L-Arg (N-DNS-Phe- Leu-Arg) as substrates [21].

In this paper, we describe a new and sensitive assay for cathepsin A activity using 5-dimethylaminonaphthalene-1-sulfonyl-L-Phe-L-Leu (N-DNS-Phe-Leu) as substrate by HPLC on a reversed-phase column to achieve a rapid and selective separation of substrate and product. This system possesses several distinct advantages when compared with the above procedures and, therefore, is suitable for a routine assay of cathepsin A activity. This assay may also be useful for studying the physiological role of cathepsin A.

2. Experimental

2.1. Chemicals

Soybean trypsin inhibitor, dithiothreitol, bacitracin, ascorbic acid and 1,10-phenanthroline monohydrate were purchased from Wako (Tokyo, Japan). 5-Dimethylaminonaphthalene-1-sulfonyl chloride (N-DNS-chloride), 5-dimethylaminonaphthalene-1-sulfonyl-L-phenylalanine free acid (N-DNS-Phe), 5-dimethylaminonaphthalene-1-sulfonyl-DL-norleucine cyclohexylammonium salt (N-DNS-NLeu), *N*-ethylmaleimide (NEM), phenylmethylsulfonyl fluoride (PMSF), pepstatin A, *p*-chloromercuriphenylsulfonic acid (PCMS), diisopropylfluorophosphate (DFP), iodoacetic acid (IAA), β-nicotinamide adenine dinucleotide (β-NAD), β-nicotinamide adenine dinucleotide, reduced form (β-NADH), β-nicotinamide adenine dinucleotide phosphate (β-NADP), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), flavine adenine dinucleotide (FAD), flavine mononucleotide (FMN), reduced gluthathione and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). L-Phe-L-Leu was from Bachem Feinchemikalien (Bubendorf, Switzerland). 1-Heptanesulfonic acid sodium salt was from Aldrich (Milwaukee, WI, USA). Acetonitrile was of chromatographic grade (Wako). Other chemicals and solvents were of analytical reagent grade.

2.2. Preparation of enzyme source

Male ICR mice weighting 20–25 g were purchased from Charles River (Japan) and housed on a 12-h light–dark cycle for at least 1 week before the beginning of all experiments. Food and water were available ad libitum. All operations were carried out at 0–4°C unless stated otherwise. Mice were killed by decapitation. After washing the whole kidney with saline, it was cut into small pieces and homogenized in nine volumes of 0.25 *M* sucrose with a glass-PTFE homogenizer. The homogenate

was centrifuged at 100 000 *g* for 80 min and the supernatant obtained was used as an enzyme source.

For the investigation on localization of enzyme activity, various organs were dissected on ice. Tissues were homogenized in nine volumes of 0.25 *M* and/or 0.32 *M* sucrose with a glass–PTFE homogenizer. The homogenates were used as an enzyme source.

2.3. Peptide synthesis

N-DNS-Phe-Leu was synthesized by the method of Wiedmeier et al. [22] with minor modifications. In brief, 169 μmol of L-Phe-L-Leu was dissolved in 10 ml of 100 m*M* sodium carbonate–sodium bicarbonate buffer (pH 9.6). To this peptide solution, 34 μmol of N-DNS-chloride in 10 ml of acetone was added. The reaction mixture was stirred at room temperature for 5 h, and thereafter the acetone was evaporated under reduced pressure. The synthetic dansylated product was purified by reversed-phase HPLC on a TSK gel ODS-PREP column (300×7.8 mm I.D., particle size, 10 μm, TOSOH) before use [23]. Following purification, the structure was confirmed by NMR and IR.

2.4. Assay for cathepsin A activity

The principle of the assay for cathepsin A activity is based on the fluorimetric measurement of N-DNS-Phe liberated enzymatically from the substrate, N-DNS-Phe-Leu, after separation by HPLC. The reaction mixture contained 50 m*M* sodium acetate buffer (pH 4.6), 40 μ*M* N-DNS-Phe-Leu, and enzyme plus water in a total reaction volume of 250 μl. Incubation was carried out at 37°C, and the reaction was terminated by heating at 95°C for 5 min in boiling water. After centrifugation, N-DNS-NLeu was added to clear supernatant as the internal standard, and an aliquot of the mixture obtained was subjected to HPLC analysis. The peak height of N-DNS-Phe was measured and converted into picomoles from the peak height of N-DNS-NLeu added as an internal standard. One unit of enzyme activity is defined as the amount of enzyme requires to convert 1 pmol of the substrate into the corresponding product in 1 min at 37°C.

2.5. Chromatographic conditions

Analysis of the product was performed using a Shimadzu (Kyoto, Japan) HPLC system consisting of a LC-10AD pump, RF-10A fluorescence detector, CTO-10A column oven, DGU-12A degasser, SCL-10A system controller and C-R6A Chromatopac. The system was operated at 35°C at a flow-rate of 1.0 ml/min employing a TSK gel ODS-80TM (particle size, 5 μm) reversed-phase column (150×4.6 mm I.D.) fitted with a TSK guard gel ODS-80TM (15 ×3.2 mm I.D., particle size, 5 μm). The mobile phase consisted of 10 m*M* sodium acetate buffer containing 0.1% 1-heptanesulfonic acid sodium salt (pH 4.2)–acetonitrile (49:51, v/v). The fluorescence was monitored with excitation at 330 nm and emission at 458 nm.

2.6. Protein determination

Protein concentration was measured by the Lowry method as modified by Hartree [24] using BSA as standard protein.

3. Results

Stock solutions of N-DNS-Phe-Leu, N-DNS-Phe and N-DNS-NLeu were stable for at least 3 months at −20°C. This HPLC–fluorimetric detection system for the measurement of N-DNS-Phe-Leu and N-DNS-Phe was found to be very sensitive. The calibration graph for N-DNS-Phe injected showed good linearity from 300 fmol to 700 pmol. The calibration graph for N-DNS-NLeu also showed good linearity from 300 fmol to 1000 pmol. Fig. 1 shows the chromatographic patterns of the reaction mixture after incubation with 6.34 μg of protein prepared from mouse kidney supernatant for 60 min. The blank incubation (Fig. 1A) contained N-DNS-Phe-Leu and N-DNS-NLeu, and the standard incubation contained exogenous N-DNS-Phe in addition to N-DNS-Phe-Leu and N-DNS-NLeu (Fig. 1B). The retention times for N-DNS-Phe, N-DNS-NLeu and N-DNS-Phe-Leu were 3.5, 4.1 and 6.2 min, respectively (Fig. 1A and 1B). The experimental incubation under the standard assay conditions (Fig. 1C) produced a significant amount of N-DNS-Phe in 3.5

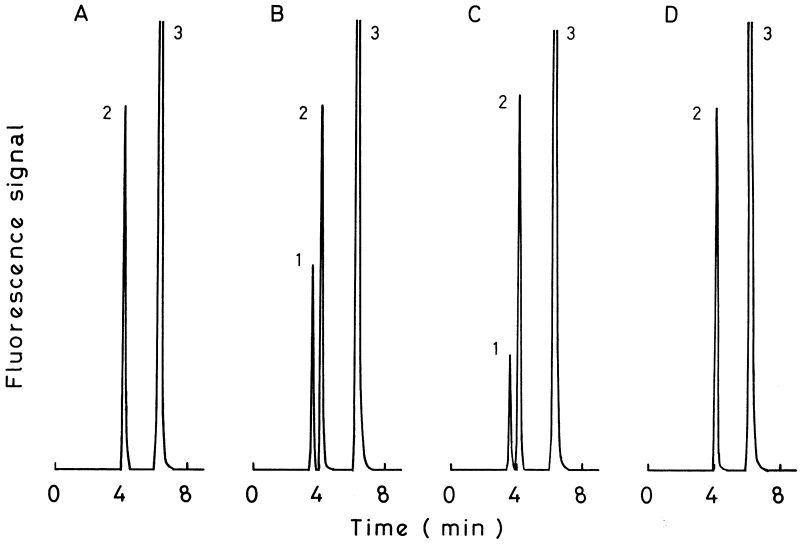

Fig. 1. HPLC elution patterns of cathepsin A activity determined using enzyme in mouse kidney. Conditions are described in Experimental. Peaks: 1, N-DNS-Phe; 2, N-DNS-NLeu; 3, N-DNS-Phe-Leu. Five hundred picomoles of N-DNS-NLeu (internal standard) was added to each sample after incubation. The enzyme activity was expressed as pmol/μg protein/h. (A) Blank incubation: N-DNS-Phe-Leu was incubated without enzyme at 37°C for 60 min. (B) Standard incubation: 500 pmol of N-DNS-Phe was added to the sample tube before incubation as a standard sample. The two peak heights of N-DNS-Phe and N-DNS-NLeu correspond to 50 pmol. (C) Incubation: N-DNS-Phe-Leu was incubated with 6.34 μg of protein in mouse kidney extract at 37°C for 60 min. (D) Control incubation: a control tube without the enzyme was incubated, the same amount of active enzyme was added, and the resulting tube was kept in an ice bath before heating at 95°C for 5 min.

min, whereas the control incubation did not show any peak of N-DNS-Phe (Fig. 1D).

The enzyme reaction was found to be linear with time at 37°C at least for about 60 min (data not shown).

The pH dependence of enzyme activity was investigated in 50 m*M* sodium acetate buffer (pH 3.4–6.4). The catalytic activity of the enzyme was greatest at a pH of approximately 4.5–5.0, with very little activity below pH 3.6 and above pH 5.6 (Fig. 2).

Cathepsin A activity was investigated as a function of the amount of enzyme extract obtained from mouse kidney. Perfect linearity was observed for plots of the amount of N-DNS-Phe, at least from 0.5 to 21.0 units, formed enzymatically from N-DNS-Phe-Leu against those of enzyme (data no shown).

Various reduced and oxidized flavin and pyridine nucleotides (FAD, FMN, β-NADPH, β-NADP, β-NADH, β-NAD), as well as ascorbate and reduced glutathione, were examined for their ability to stimulate the cathepsin A activity. All of the cofactors tested had no stimulating effect on the cathepsin A activity (data not shown).

A Lineweaver–Burk plot was obtained from the effect of the concentration of N-DNS-Phe-Leu on the

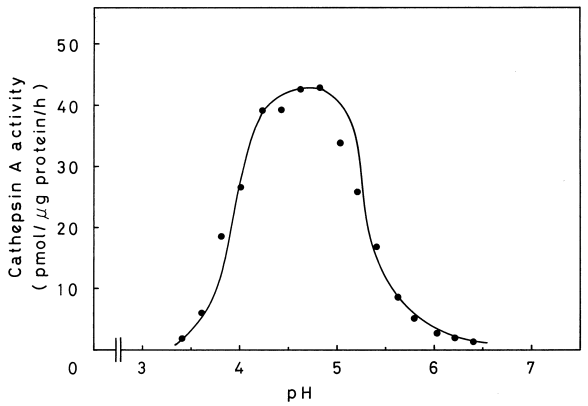

Fig. 2. Effects of pH on cathepsin A activity in mouse kidney: 50 m*M* sodium acetate buffer (pH 3.4–6.4) was used. Incubation was carried out at 37°C for 60 min. The enzyme activity was expressed as pmol/μg protein/h.

rate of formation of N-DNS-Phe by cathepsin A. The Michaelis constant (K_m) and the maximum velocity (V_{max}) toward the N-DNS-Phe-Leu were calculated to be 14.9 μ*M* and 27.91 pmol/μg/h, respectively.

We applied this standard assay to the determination of the effects of various metal ions and protease inhibitors on cathepsin A activity in mouse kidney extract. As shown in Table 1, metals such as Ag^{+} and Hg^{2+} completely inhibited the enzyme activity at 0.1 m*M*, whereas Mn^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+} had no effect. DFP (serine protease inhibitor) and PCMS (thiol protease inhibitor) inhibited it strongly at a final concentration of 1 mM. However, there was a partial inhibition by other thiol protease inhibitors such as IAA, NEM and PMSF (serine protease inhibitor). Cathepsin A activity was also partially affected by Cu^{2+} and bacitracin. On the other hand, the enzyme activity was not affected by other reagents (EDTA, 1,10-phenanthroline, dithiothreitol, soybean trypsin inhibitor and pepstatin A).

Finally, we examined the distribution of cathepsin A activity in various mouse organs (Table 2). The enzyme activity was determined using the homogenates obtained from spleen, brain, kidney, liver, lung and testis of a six-week-old mouse. It was found that the cathepsin A activity was distributed unevenly in

Table 1
Effects of various metal ions and inhibitors on cathepsin A activity in mouse kidney[a]

Reagent	Final concentration (m*M*)	Cathepsin A activity (% of control)
None		100
$MnCl_2$	1	104.5±1.3
$MnCl_2$	0.1	100.8±0.8
$CaCl_2$	1	102.4±3.1
$CaCl_2$	0.1	96.3± 1.4
$MgCl_2$	1	106.8± 2.3
$MgCl_2$	0.1	98.7± 0.6
$ZnSO_4$	1	103.1± 1.8
$ZnSO_4$	0.1	97.2± 1.6
$CuSO_4$	1	22.5± 2.2
$CuSO_4$	0.1	66.1± 2.5
$AgNO_3$	1	0
$AgNO_3$	0.1	0
$HgCl_2$	1	0
$HgCl_2$	0.1	0
EDTA	1	98.8± 3.3
EDTA	0.1	99.6± 1.3
1,10-Phenanthroline	1	99.4± 2.1
1,10-Phenanthroline	0.1	94.6± 0.7
Diisopropylfluorophosphate	1	0
Diisopropylfluorophosphate	0.1	25.3± 1.9
Phenylmethylsulfonyl fluoride	1	69.5± 1.7
Phenylmethylsulfonyl fluoride	0.1	86.7± 2.6
Iodoacetic acid	1	85.6± 1.2
Iodoacetic acid	0.1	91.6± 2.2
N-Ethylmaleimide	1	83.4± 0.7
N-Ethylmaleimide	0.1	93.7± 2.1
p-Chloromercuriphenyl sulfonic acid	1	11.4± 0.5
p-Chloromercuriphenyl sulfonic acid	0.1	16.2± 1.0
Dithiothreitol	1	95.5± 0.6
Dithiothreitol	0.1	91.8± 1.0
Bacitracin	50 μg/ml	71.2± 1.6
Soybean trypsin inhibitor	20 μg/ml	96.4± 1.4
Pepstatin A	20 μg/ml	99.5± 1.5

[a] Note: data are mean±SEM values determined in four separate experiments.

Table 2
Distribution of cathepsin A activity in mouse organs[a]

Organ	Cathepsin A activity (pmol/μg protein/h)
Spleen	5.40±0.26
Brain	2.02±0.05
Kidney	8.68±0.31
Liver	3.79±0.19
Lung	3.11±0.36
Testis	4.47±0.16

[a] Note: data are mean±SEM values from six animals.

mouse organs. Among the organs examined, the highest specific activity of the enzyme was found in the kidney, and the lowest in the brain. A moderate level of activity was observed in the spleen, liver, lung and testis.

4. Discussion

As pointed out in other HPLC enzymatic assays, the direct analysis of the product of enzyme action, separated from the substrate and other interfering substances, offers several advantages of earliar methods [3,5,17–19].

Herein we reported a new assay for cathepsin A activity by the HPLC–fluorimetric detection system using N-DNS-Phe-Leu as substrate. The amino acid sequence of substrate, Phe-Leu, was selected because it has been used by several laboratories as the most commonly used assay system for detecting cathepsin A activity [7,17,20,25,26]. The proposed sensitive assay for cathepsin A activity has a few advantages. First, it is very sensitive. The limit of the sensitivity was about 300 fmol of N-DNS-Phe formed enzymatically. Second, the substrate and the product are separated completely in less than 7.0 min. Third, more accurate quantitation of the product and better reproducibility were guaranteed in our method by the employment of internal standard (N-DNS-NLeu) compared to those of the HPLC–spectrophotometric assay system using Z-Glu-Tyr as substrate by gradient elution, in which an internal standard had not been used [10].

Moreover, we described a few physicochemical properties of cathepsin A in mouse kidney. The optimum pH of cathepsin A from mouse kidney is 4.5–5.0. The effects of various chemical reagents and protease inhibitors on cathepsin A activity are also investigated (Table 1). The cathepsin A activity in mouse kidney is completely inhibited by metals such as Ag^{+} and Hg^{2+}, but not by other metals (Mn^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+}), chelating agents (EDTA and 1,10-phenanthroline). Furthermore, the enzyme activity was also inhibited by typical serine protease inhibitor (DFP) and thiol protease inhibitor (PCMS) at a final concentration of 1 mM. The optimum pH and inhibition pattern on cathepsin A activity described above is very similar to those of the enzymes that have been isolated from other tissues [2,5,27,28].

Kase et al. [26] demonstrated simultaneous deficiency of esterase, carboxy-terminal deamidase, and acid carboxypeptidase activities together with the 'protective' activity against lysosomal β-galactosidase and neuraminidase in fibroblasts derived from patients with galactosialidosis. If cathepsin A is identical with the multifunctional enzyme protein (lysosomal protective protein) as reported previously [2], further considerations should be made to clarify the relations between its functional aspects and enzymatic characterizations.

In conclusion, the sensitive and accurate assay for cathepsin A activity described in this paper may be useful for the above mentioned purpose.

References

[1] H.H. Tallan, M.E. Jones, J.S. Fruton, J. Biol. Chem. 194 (1952) 793.
[2] H.L. Jackman, F. Tan, H. Tamei, C. Beurling-Harbury, X.-Y. Li, R.A. Skidgel, E.G. Erdos, J. Biol. Chem. 265 (1990) 11265.
[3] K. Matsuda, J. Biochem. 80 (1976) 659.
[4] Y. Kawamura, T. Matoba, T. Hata, E. Doi, J. Biochem. 81 (1977) 435.
[5] A. Grynbaum, N. Marks, J. Neurochem. 26 (1976) 313.
[6] H.L. Jackman, P.W. Morris, P.A. Deddish, R.A. Skidgel, E.G. Erdos, J. Biol. Chem. 267 (1992) 2872.
[7] K. Itoh, R. Kase, M. Shimmoto, A. Satake, H. Sakuraba, Y. Suzuki, J. Biol. Chem. 270 (1995) 515.
[8] E. Kominami, S. Hashida, N. Katsunuma, Biochim. Biophys. Acta 659 (1981) 390.
[9] N. Marks, L. Sachs, F. Stern, Peptides 2 (1981) 159.
[10] J.J. Miller, D.G. Changaris, R.S. Levy, Biochem. Biophys. Res. Commun. 154 (1988) 1122.
[11] A. D'Azzo, A. Hoogeveen, A.J.J. Reuser, D. Robinson, H. Galjaard, Proc. Natl. Acad. Sci. USA 79 (1982) 4535.

[12] A.T. Hoogeveen, F.W. Verheijen, H. Galjaard, J. Biol. Chem. 258 (1983) 12143.
[13] F.W. Verheijen, S. Palmeri, A.T. Hoogeveen, H. Galjaard, Eur. J. Biochem. 149 (1985) 315.
[14] E. Nanba, A. Tsuji, K. Omura, Y. Suzuki, Biochem. Biophys. Res. Commun. 144 (1987) 138.
[15] Y. Suzuki, H. Sakuraba, T. Yamanaka, Y. Y-.M. Ko, Y. IImori, A.T. Okamura, in: M. Arima, Y. Suzuki, H. Yabuuchi (Eds.), The Developing Brain and Its Disorders, Karger, Basel, 1985, pp. 161–175.
[16] J.S. O'Brien, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic Basis of Inherited Disease II, 6th ed, McGraw-Hill, New York, 1989, pp. 1797–1806.
[17] T.H. Stevens, J.H. Rothman, G.S. Payne, R. Schekman, J. Cell Biol. 102 (1986) 1551.
[18] S. Taylor, A.L. Tappel, Anal. Biochem. 56 (1973) 140.
[19] W.J. Kleijer, G.C. Geilen, H.C. Janse, O.P. Van Diggelen, X.Y. Zhou, N.J. Galjart, H. Galjaard, A. D'Azzo, Pediatr. Res. 39 (1996) 1067.
[20] A.V. Pshezhetsky, M.V. Vinogradova, M.-A. Elsliger, F. El-Zein, V.K. Svedas, M. Potier, Anal. Biochem. 230 (1995) 303.
[21] P.A. Deddish, R.A. Skidgel, V.B. Kriho, X.-Y. Li, R.P. Becker, E.G. Erdos, J. Biol. Chem. 265 (1990) 15083.
[22] V.T. Wiedmeier, S.P. Porterfield, C.E. Hendrich, J. Chromatogr. 231 (1982) 410.
[23] T. Chikuma, K. Hanaoka, Y.P. Loh, T. Kato, Y. Ishii, Anal. Biochem. 198 (1991) 263.
[24] E.F. Hartree, Anal. Biochem. 48 (1972) 422.
[25] J. Tranchemontagne, L. Michaud, M. Potier, Biochem. Biophys. Res. Commun. 168 (1990) 22.
[26] R. Kase, K. Itoh, N. Takiyama, A. Oshima, H. Sakuraba, Y. Suzuki, Biochem. Biophys. Res. Commun. 172 (1990) 1175.
[27] J.K. McDonald, A.J. Barrett, in: 2nd ed, Mammalian Proteases, A Glossary and Bibliography, Vol. 2, Academic Press, New York, 1986, pp. 186–191.
[28] J.J. Miller, D.G. Changaris, R.S. Levy, J. Chromatogr. 627 (1992) 153.