Cathepsin D is specifically inhibited by deoxyribonucleic acids

Masahiro Shibata, Masato Koike, Satoshi Waguri, Guiqin Zhang, Tomoyuki Koga, Yasuo Uchiyama*

Department of Cell Biology and Neuroscience, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Received 13 December 2001; revised 4 March 2002; accepted 5 March 2002

First published online 5 April 2002

Edited by Judit Ovádi

Abstract A cathepsin D (CD) inhibitor was searched using mouse embryonic fibroblasts deficient for CD. Synthetic DNA fragments specifically inhibited CD activity in a dose-dependent manner, but not the activities of other serine or cysteine proteinases. Cathepsin E activity was also inhibited by DNA fragments when hemoglobin was used as a substrate. CD inhibition by DNA fragments appeared to be electrostatic in nature and dependent on T_m values. Moreover, CD activity was partly inhibited by exogenously ingested DNA fragments, suggesting that DNA fragments with high T_m values are potent inhibitors of CD in vitro and partly in vivo.

Key words: Inhibitor; Cathepsin D; DNA; Mouse embryonic fibroblast

1. Introduction

Lysosomes, ubiquitous in all animal cells as an acidic compartment with limiting membranes are able to degrade unneeded intra- and extracellular materials to biological monomers and contain various types of proteinases except for metalloproteinases. Most lysosomal proteinases are cysteine proteinases, while cystatins which are known to be present in intra- and extracellular milieu, act as cysteine proteinase inhibitors [1]. No specific inhibitors of aspartic and serine proteinases in lysosomes have yet been found, although serpins, an inhibitor family of serine proteinases, are present [2].

Cathepsin D (CD), a representative aspartic proteinase in lysosomes, is widely distributed in tissue cells and has been shown to be involved in aging and certain pathological situations in brain tissues [3,4]. A study of CD deficient (CD−/−) mice has shown that CD plays a crucial role in the degradation of cellular metabolites especially in neurons of the central nervous system [5–7]. However, little is known about the presence of endogenous inhibitors in mammalian cells.

In the course of a study to determine whether an endogenous inhibitor of CD is present in mammalian cells using CD−/− mouse embryonic fibroblasts (MEFCD−/−), we noted that the activity of purified CD is significantly reduced when measured in the presence of extracts of MEFCD−/−, indicating that some inhibiting substance is present in these extracts. In the present study, we report an analysis of MEFCD−/− to identify the specific inhibitor of CD. We conclude that CD is specifically inhibited by deoxyribonucleic acids whose inhibition is dependent on the T_m value of the nucleic acids.

2. Materials and methods

2.1. Enzymes and reagents

Bovine trypsin, chymotrypsin, and cathepsins B and D were obtained from Sigma (St. Louis, MO, USA). Cathepsin E (CE) was purified from rat stomach as described previously [8]. Oligo- and polynucleotides were of various lengths (6–53 bases) and T_m values (0–100°C) including DNA_{18b} (5′-ggctcaggtacccagaga-3′) and DNA_{53b} (5′-gctctagatcaatgatgatgatgatgctggcgtttccgaggggggcgctt-3′), obtained from Invitrogen (Carlsbad, CA, USA). The following substrates and reference compounds were obtained from the Peptide Institute (Osaka, Japan): *t*-butoxycarbonyl-Gln-Ala-Arg-methylcoumarinamide (MCA) for trypsin, succinyl-Ala-Ala-Pro-Phe-MCA for chymotrypsin, *z*-Arg-Arg-MCA for cathepsin B, Arg-MCA for cathepsin H, Phe-Arg-MCA for cathepsin L, Ala-Ala-Phe-MCA for tripeptidyl peptidase-I (TPP-I), 7-methoxycoumarin-4-yl-acetyl (MOCAc)-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (Dnp)-$_D$-Arg-NH_2 for CD and CE, and 7-amino-4-methyl-coumarin- and MOCAc-Pro-Leu-Gly for reference compounds. Denatured bovine hemoglobin (Wako Pure Chemical Ind., Inc., Osaka, Japan) was used for a substrate for CD after being labeled with $NaB[^3H]_4$ (Perkin-Elmer Life Sciences Inc., Boston, MA, USA) according to the method described previously [9]. A substrate, Gly-Phe-pNA, for cathepsin C was obtained from Sigma.

2.2. Enzyme assay

Measurements of proteolytic activity of CD and CE were performed in a reaction buffer: 50 mM sodium acetate buffer, pH 4.0, containing 10 μM of the substrate and 1 μg/ml of leupeptin, by incubation at 37°C in the presence or absence of various types of nucleic acids. The fluorescence intensity of reacted samples at 390 nm (Ex = 330 nm) was monitored for 15 min at 30 s intervals using a Biolumin™ 960 (Amersham Biosciences AB, Uppsala, Sweden), while the initial rate was determined from the initial velocity (v) using MOCAc-Pro-Leu-Gly as a reference compound. The proteolytic activity of these enzymes was also measured using a ^3H-labeled hemoglobin according to the modified method by Anson [10]. Enzyme–substrate mixtures were incubated in the reaction buffer with or without various concentrations of DNA_{18b}, similar to the above mentioned procedure, and the reaction was stopped by the addition of 10% trichloroacetic acid. After centrifugation, radioactivity in supernatants was counted. The inhibition activity of CD by nucleic acids was also measured at various concentrations of NaCl. The proteolytic activity of trypsin and chymotrypsin, and lysosomal cathepsins B, C, H, and L, and TPP-I was also assayed, as described previously [11–15]. For measurements of inhibition rates of lysosomal proteinases with various con-

*Corresponding author. Fax: (81)-6-6879-3129.
E-mail address: uchiyama@anat1.med.osaka-u.ac.jp (Y. Uchiyama).

Abbreviations: CD, cathepsin D; CE, cathepsin E; DNA_{18b}, 5′-ggctcaggtacccagaga-3′; DNA_{53b}, 5′-gctctagatcaatgatgatgatgatgctggcgtttccgaggggggcgctt-3′; MCA, methylcoumarinamide; MOCAc, 7-methoxycoumarin-4-yl-acetyl; MEF, mouse embryonic fibroblasts; CD−/−, deficient for CD; TPP-I, tripeptidyl peptidase-I; wt, wild-type

PII: S0014-5793(02)02569-3

centrations of DNA_{18b}, lysosome-rich fractions were obtained from mouse livers, as described previously [16].

2.3. Cell culture and transfection

MEFCD−/− and wild-type (wt) MEF (kind gift from Dr. Paul Saftig, Göttingen, Germany) were cultured in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin sulfate, and 100 U/ml penicillin. The cells were seeded 24 h prior to the transfection on a 12 mm cover glass (Matsunami Glass Ind., Ltd., Tokyo, Japan). DNA_{18b} was labeled with biotin-16-dUTP (F. Hoffmann, La Roche Ltd., Basel, Switzerland) using a terminal deoxynucleotidyl transferase (TOYOBO, Tokyo, Japan). Labeled DNA_{18b} was transfected into wt MEF by the calcium phosphate precipitation method.

To confirm the intracellular localization of labeled DNA_{18b}, the cells were fixed with 4% paraformaldehyde buffered with 0.1 M phosphate buffer, pH 7.4, and then doubly stained with avidin–Texas red (Nichirei, Tokyo, Japan) and a monoclonal antibody to rat LAMP2 (Developmental Studies Hybridoma Bank, University of Iowa, USA), a lysosomal membrane protein, at 4°C overnight. They were further reacted with anti-rat IgG coupled with FITC (Organon Teknika Corp., PA, USA) for 1 h and observed by confocal laser microscopy (LSM-25, Olympus, Tokyo, Japan).

2.4. Purification of CD inhibitor

MEFCD−/− harvested from cultures were lysed with a lysis buffer consisting of 0.15 M NaCl, 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 1% Triton X-100, and the lysates were centrifuged at $15000\times g$ for 10 min. The resultant supernatants were applied onto a SP–Sepharose (cationic exchange) column equilibrated with 20 mM Tris–HCl buffer, pH 7.0. After washing the column with the same buffer, inhibiting activity of CD eluted with a linear gradient of 0–1 M NaCl. Fractions showing inhibiting activity of CD were pooled, adjusted with phosphate buffer to a final concentration of 20 mM phosphate, pH 7.0, containing 1 M ammonium sulfate, and then, applied onto a phenyl–Sepharose (hydrophobic interaction) column equilibrated with the same buffer. After washing the column with the same buffer, the inhibiting substance eluted with a linear gradient of 1–0 M $(NH_4)_2SO_4$. The active fractions collected were applied onto a Sephacryl H-200R (gel filtration) column equilibrated with 20 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl and eluted. The active fractions were pooled and stored at −80°C until used.

2.5. Subcellular fractionation

The cells transfected with or without DNA_{18b} were washed with phosphate-buffered saline and homogenized in 0.25 M sucrose by passing through a 27 gauge needle several times. Lysosome-rich fractions from the homogenates were obtained by previously described methods [16]. One part of the lysosome-rich fractions was assayed for the CD activity and the other was analyzed by Western blotting to determine the protein level of CD in the lysosomal fractions by measuring the blotted density with a scanning imager (Amersham Biosciences AB).

3. Results and discussion

3.1. Purification and characterization of CD inhibitor

The activity of purified bovine CD which was measured in the presence of lysates from MEFCD−/− or the buffer solution, was reduced when lysates of MEFCD−/− were present. Cationic exchange chromatography, hydrophobic interaction chromatography, and gel filtration were performed to purify a potent CD inhibitor, based on activity measurements. The inhibiting substance was not detected by SDS–PAGE followed by silver staining, but was stained by ethidium bromide (Fig. 1A). The fraction containing the active substance eluted near the void volume during gel filtration, where no protein was detected. These results indicate that the inhibiting substance of CD activity is a nucleic acid.

Using DNA_{18b} and DNA_{53b} as model DNA fragments, their ability to inhibit lysosomal proteinases was tested in lysosome-rich fractions. The activity of lysosomal cysteine and serine proteinases including cathepsins B, C, H, and L, and TPP-I was not inhibited by DNA_{18b}, although CD activity was largely inhibited (Fig. 1B). The inhibition of trypsin and chymotrypsin, serine proteinases, by these DNA fragments was examined, but no inhibition was detected (data not shown). Using a fluorogenic substrate, the inhibition of the bovine CD activity by DNA_{18b} was then analyzed and found to occur in a dose-dependent manner: a 76.1% inhibition by 100 nM of DNA_{18b} (Fig. 1C). The complete inhibition by DNA_{18b} required a concentration of 10 μM, while 1 μM of DNA_{53b} completely suppressed CD activity (Fig. 1C). The inhibitory effect of DNA_{18b} on CD activity was also examined when ^{3}H-labeled hemoglobin was used as a substrate, and was found to be similar to that obtained by using the fluorogenic substrate (data not shown). The inhibition by DNAs occurred at a physiological salt concentration (0.15 M NaCl), but an increase in ionic strength led to a decrease in inhibition: concentrations of DNA_{18b} and plasmid DNA required for a 50% inhibition of CD activity (IC50) at 0.3 M NaCl were 1.7×10^{-5} M and 1.5×10^{-9} M, respectively, while a concentration of NaCl to give IC50 by 1×10^{-7} M of DNA_{18b} was 0.276 M.

Since the enzymatic characteristics of CD are similar to those of CE, DNA_{18b} or DNA_{53b} was applied to the enzyme assay of CE obtained from rat stomach. CE activity was not inhibited by DNAs, but was rather enhanced, compared to that incubated without DNAs, when the fluorogenic substrate was used (Fig. 1D). Similar to the case of CD activity, how-

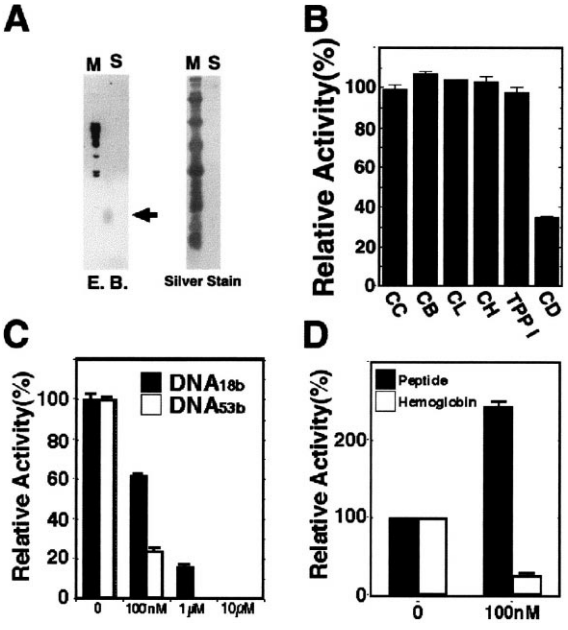

Fig. 1. Inhibition of CD by nucleic acids. A: A purified inhibitory substance (S) was subjected to 0.7% agarose gel electrophoresis (left panel) and 12.5% SDS–PAGE (right panel), followed by ethidium bromide (E.B.) and silver staining, respectively. M are marker DNAs in the left panel and low range marker proteins in right panel. Arrow indicates a purified DNA fragment. B: DNA_{18b} at a concentration of 100 nM does not inhibit the proteolytic activities of cathepsins B (CB), C (CC), H (CH), and L (CL), and TPP-I in lysosome-rich fractions, except for that of CD. C: Dose-dependent inhibition of bovine CD activity by DNA_{18b} and DNA_{53b}. D: Different effects of DNA_{18b} at a concentration of 100 nM on proteolytic activity of CE between a fluorogenic substrate (peptide) and ^{3}H-labeled hemoglobin. Vertical bars indicate the standard deviation.

Table 1
Inhibition of CD activity by various types of DNA

DNA	Length	Strand	IC50 (M)
Plasmid	55 kbp	double	6×10^{-13}
Oligonucleotide	18 mer	single	5×10^{-8}
dNTPs	1 base	single	N.D.
E. coli genome	4.7 Mbp	double	8×10^{-14}

The concentration of CD used is 2.5 nM. The oligonucleotide sequence used is 5′-ggctcaggtacccagaga-3′. N.D., not detectable.

ever, CE activity was inhibited by DNA_{18b} when ^3H-labeled hemoglobin was used as a substrate (Fig. 1D).

3.2. CD activity is inhibited by various deoxyribonucleic acids

To determine the characteristics of deoxyribonucleic acids that inhibit CD, various types of DNAs were prepared and their inhibiting activity examined. As shown in Table 1, inhibition of CD activity was observed by a plasmid DNA (pcDNA3), DNA_{18b} and *Escherichia coli* genomic DNA. IC50 differed depending on the DNA masses: larger DNA masses required lower IC50 values: *E. coli* genome (4.7 Mbp) > plasmid DNA (5.5 kbp) > DNA_{18b}. Inhibition of CD activity was detected by both single-stranded and double-stranded DNAs, while dNTPs, a mixture of monomeric DNAs, showed no inhibition. For inhibiting CD activity, the DNA fragments were required to have at least 10 bases, the inhibition rate of which was about 40% at a concentration of 100 nM (data not shown).

Since CD activity tended to be inhibited by DNAs of larger masses, their characteristics were examined further. For this, various sorts of synthetic oligonucleotides with different length (from 18 to 53 bases), GC content (0–100%), and T_m values (36–75°C) were prepared. CD activity was measured in the presence of 100 nM of each oligonucleotide; in each reaction the initial velocity (v) was determined and the inhibitory rates (%) calculated (Fig. 2). The inhibiting activity was not simply related to GC content (Fig. 2A) and the length of oligonucleotides (Fig. 2B), but was significantly dependent on the T_m values of each nucleotide (Fig. 2C). The results suggest that the intensity of inhibition by oligonucleotides is correlated to both GC content and length.

3.3. Endocytosed DNA inhibits CD activity

To examine the issue of whether CD activity is inhibited by exogenously administered nucleic acids, DNA_{18b} was transfected into wt MEF. Transfected DNA was detected by double staining in a granular form and largely co-localized with LAMP2, a lysosomal membrane marker protein in MEF cytoplasm (Fig. 3A). To compare the amounts of CD protein and its activity, lysosomal fractions were obtained from MEF before and 12 h after transfection. Although the protein levels of CD in the cells were similar (Fig. 3B), the specific activity of CD was significantly reduced in the transfected cells (Fig. 3C). These results suggest that the CD activity is also suppressed by exogenous DNA.

It has been shown that polynucleotides such as yeast transfer RNA and calf thymus DNA inhibit the activity of human leucocyte elastase, and that this inhibition is dependent on ionic strength [17,18]. Moreover, DNA fragments interfere with the inhibition of human neutrophil-derived cathepsin G by lung tissue inhibitors, while the inhibition of elastase by the inhibitors is much less than that of cathepsin G [19]. The difference in inhibition rates of these proteinases may be attributed to the presence of cationic amino acid residues, such as arginine, in the vicinity of their active centers [20,21].

The inhibition of the CD activity by DNA_{18b} or DNA_{53b} was dependent on ionic strength. The content of cationic amino acid residues such as arginine and lysine is high in CD: 8.5% in human CD, 9.3% in rat and mouse CD, and 9% in bovine CD, indicating that the binding of CD to DNAs may be due to ionic reactions. The inhibition of CE activity by the DNA fragments was also analyzed, since CD and CE are

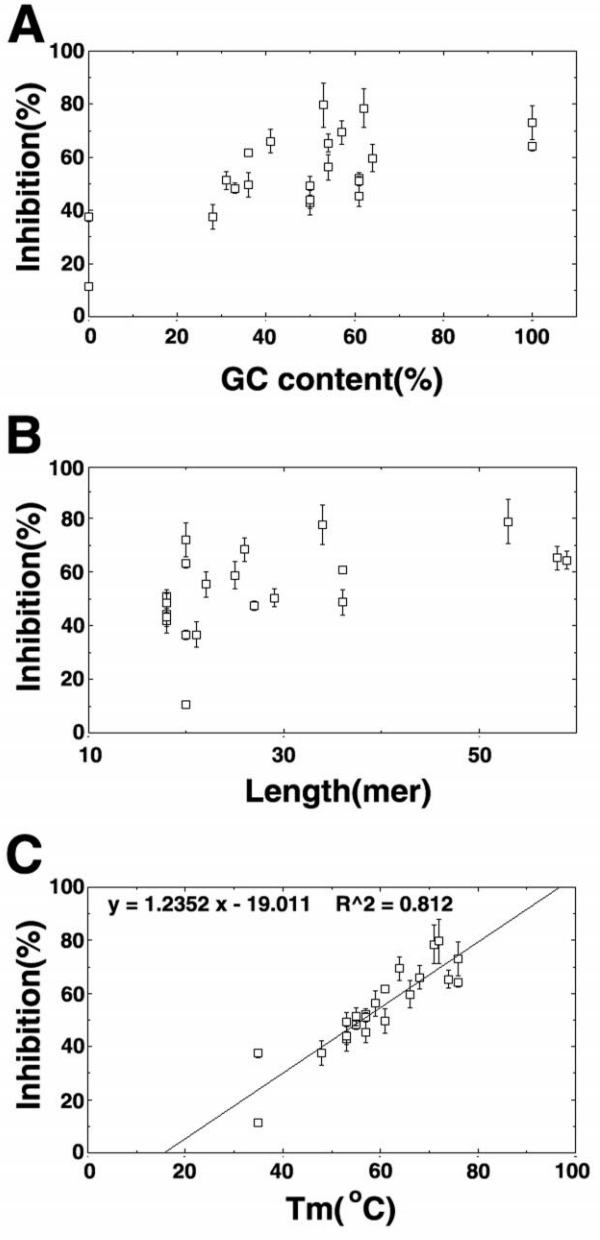

Fig. 2. Correlation of inhibitory rates of CD activity and various synthetic DNA fragments. A,B: Correlation between inhibitory rates (%) and GC content (A) or length (B). C: Correlation between the inhibitory rates (%) and T_m values which are calculated from the equation $60.8+0.41\times$(GC content (%))$-500/$(the number of nucleotides). Plotted data show a significant correlation between the two parameters and is expressed by an indicated formula as a linear regression line. All assays were performed at least three times. Vertical bars indicate the standard deviation.

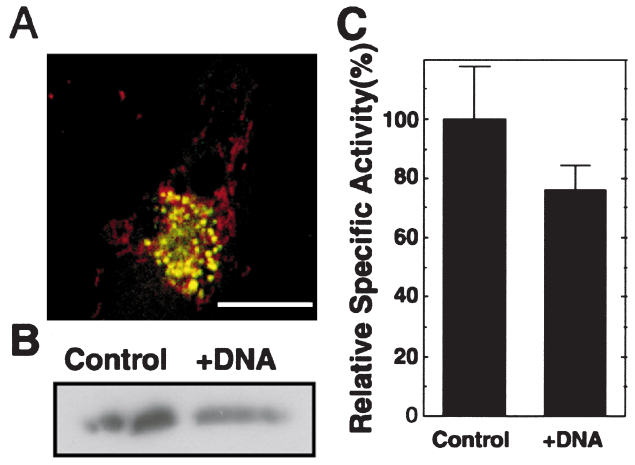

Fig. 3. Inhibition of CD activity in wt MEF by transfected DNA_{18b}. A: Double staining of biotinylated DNA_{18b} and LAMP2, a lysosomal membrane protein, in MEF 12 h after transfection. Yellow color indicates the co-localization of DNA_{18b} with LAMP2 in the cell. Bar indicates 10 μm. B: CD protein bands in lysosome-rich fractions obtained from wt MEF (control) and the cells 12 h after transfection with DNA_{18b} by Western blot. C: Relative activity of CD in lysosome-rich fractions obtained from control and DNA_{18b}-transfected cells. All assays were performed at least three times. Vertical bars indicate the standard deviation.

biochemically and catalytically similar [8,22]. The inhibitory effect of DNA fragments on CE activity differed depending on substrates used: CE activity was rather enhanced when the fluorogenic substrate was used, but it was inhibited when ^{3}H-labeled hemoglobin was used. It is supposed that the electrostatic complexes of DNA fragments and CD or CE do not hinder all enzyme activity, but have a certain residual activity. In the case of CD activity, this residual enzyme activity may be small for both a synthetic substrate and hemoglobin, since the inhibitory effect of DNA fragments did not differ between substrates used. In the case of CE activity, however, the residual activity may be enough for a small synthetic substrate, but not for hemoglobin with a larger molecular size.

A variety of inhibitors of aspartic proteinases in plant, ascaris and yeast are known [23–25], but most inhibitors are effective on aspartic proteinases, except for the ascaris inhibitor which is specific to pepsin and CE [24]. To date, however, no reports have appeared on a specific inhibitor of CD in mammalian cells and tissues, except for this report. Similar to the ascaris inhibitor, DNAs with high T_m values are potent inhibitors of CD.

The present study shows that transfected DNA_{18b} partially inhibits the CD activity in MEF. Phagocytic cells such as macrophages and microglial cells often ingest cell debris derived from necrosis and apoptosis at inflammatory or damaged tissue sites, suggesting the possibility that the CD activity in these phagocytes is inhibited by ingested DNAs.

Moreover, we have previously shown that CD deficiency induces the accumulation of ceroid-lipofuscin in neurons of the central nervous system and iNOS in microglial cells and macrophages which leads to neuronal and small intestinal epithelial cell death [6,7]. These lines of evidence suggest that phagocytic cells which ingest DNAs may partly contribute to the production of cytocidal cytokines such as NO via iNOS activity in inflammatory and damaged tissue sites.

Thus, DNAs with high T_m values are potent inhibitors of CD in vitro and partly in vivo.

Acknowledgements: We thank Dr. Paul Saftig (Göttingen University, Germany) for giving us wt MEF and MEFCD−/−.

References

[1] Brown, W.M. and Dziegielewska, K.M. (1997) Protein Sci. 6, 5–12.
[2] Potempa, J., Korzus, E. and Travis, J. (1994) J. Biol. Chem. 269, 15957–15960.
[3] Nakanishi, H., Amano, T., Sastradipura, D.F., Yoshimine, Y., Tsukuba, T., Tanabe, K., Hirotsu, I., Ohono, T. and Yamamoto, K. (1997) J. Neurochem. 68, 739–749.
[4] Cataldo, A.M., Barnett, J.L., Berman, S.A., Li, J., Quarless, S., Bursztajn, S., Lippa, C. and Nixon, R.A. (1995) Neuron 14, 671–680.
[5] Saftig, P., Hetman, M., Schmahl, W., Weber, K., Heine, L., Mossmann, H., Koster, A., Hess, B., Evers, M. and von Figura, K. (1995) EMBO J. 14, 3599–3608.
[6] Koike, M., Nakanishi, H., Saftig, P., Ezaki, J., Isahara, K., Ohsawa, Y., Schulz-Schaeffer, W., Watanabe, T., Waguri, S., Kametaka, S., Shibata, M., Yamamoto, K., Kominami, E., Peters, C., von Figura, K. and Uchiyama, Y. (2000) J. Neurosci. 20, 6898–6906.
[7] Nakanishi, H., Zhang, J., Koike, M., Nishioku, T., Okamoto, Y., Kominami, E., von Figura, K., Peters, C., Yamamoto, K., Saftig, P. and Uchiyama, Y. (2001) J. Neurosci. 21, 7526–7533.
[8] Takeda, M., Ueno, E., Kato, Y. and Yamamoto, K. (1986) J. Biochem. 100, 1269–1277.
[9] Birkedal-Hansen, H. and Dano, K. (1981) Anal. Biochem. 115, 18–26.
[10] Anson, M.L. (1939) J. Gen. Physiol. 22, 79–89.
[11] Kawabata, S., Miura, T., Morita, T., Kato, H., Fujikawa, K., Iwanaga, S., Takada, K., Kimura, T. and Sakakibara, S. (1988) Eur. J. Biochem. 172, 17–25.
[12] Sawada, H., Yokosawa, H., Hoshi, M. and Ishii, S. (1983) Experientia 39, 377–378.
[13] Barrett, A.J. and Kirschke, H. (1981) Methods Enzymol. 80, 535–561.
[14] Wolters, P.J., Raymond, W.W., Blount, J.L. and Caughey, G.H. (1998) J. Biol. Chem. 273, 15514–15520.
[15] Page, A.E., Fuller, K., Chambers, T.J. and Warburton, M.J. (1993) Arch. Biochem. Biophys. 306, 354–359.
[16] Ezaki, J., Wolfe, L.S., Higuti, T., Ishidoh, K. and Kominami, E. (1995) J. Neurochem. 64, 733–741.
[17] Lestienne, P. and Bieth, J.G. (1983) Biochimie 65, 49–52.
[18] Belorgey, D. and Bieth, J.G. (1995) FEBS Lett. 361, 265–268.
[19] Duranton, J., Boudier, C., Belorgey, D., Mellet, P. and Bieth, J.G. (2000) J. Biol. Chem. 275, 3787–3792.
[20] Bode, W., Wei, A.Z., Huber, R., Meyer, E., Travis, J. and Neumann, S. (1986) EMBO J. 5, 2453–2458.
[21] Hof, P., Mayr, I., Huber, R., Korzus, E., Potempa, J., Travis, J., Powers, J.C. and Bode, W. (1996) EMBO J. 15, 5481–5491.
[22] Yamamoto, K., Sakai, H., Ueno, E. and Kato, Y. (1991) Adv. Exp. Med. Biol. 306, 297–306.
[23] Mares, M., Meloun, B., Pavlik, M., Kostka, V. and Baudys, M. (1989) FEBS Lett. 251, 94–98.
[24] Kageyama, T. (1998) Eur. J. Biochem. 253, 804–809.
[25] Li, M., Phylip, L.H., Lees, W.E., Winther, J.R., Dunn, B.M., Wlodawer, A., Kay, J. and Gustchina, A. (2000) Nat. Struct. Biol. 7, 113–117.