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Biochemical and immunohistochemical study on physiological activity and distribution of hepatic cathepsin D

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Cathepsin D (EC 3.4.23.5) is a lysosomal endopeptidase physiologically present at very low concentration in different tissues. The aim of the study was to estimate the physiological activity and distribution of cathepsin D in the liver. Four groups of ten-week-old male Wistar rats were raised without xenobiotics and sacrificed on day 4, 42, 47 and 84 of the experiment, and their livers were taken for immunohistochemical and biochemical investigation. Immunostaining for cathepsin D was evaluated by light microscope. Activity of the free and bound fractions of hepatic cathepsin D was measured spectrophotometrically.

Immunohistochemical staining for cathepsin D was positive in Browicz-Kupffer cells in some but not in all rat liver specimens of each experimental group. The staining pattern was cytoplasmic and granular. Occasionally the positive stained endothelial cells were also found. No activity of cathepsin D in hepatocytes was detected. The positive immunostaining was found in livers with high enzyme activity in the biochemical investigation. No significant differences in activity of the free and bound fractions of cathepsin D among the different age groups were noted. However, the higher, age-dependent activity ($p>0.05$) of the free fraction was observed in the youngest and the two-middle groups of rats that were sacrificed on day 42 and 47 than in the oldest one. The bound fraction did not reveal such changes.

It could be concluded that there were no differences in the activity of hepatic free and bound fractions of cathepsin D in male Wistar rats of various reproductive age. The rat Browicz-Kupffer cells revealed the highest activity of cathepsin D.

Keywords: lysosomal enzymes, cathepsin D, liver, Browicz-Kupffer cell, Kupffer cell, rat

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Various experimental and clinical studies reported usefulness of the evaluation of lysosomal enzymes in the course of physiological and pathological processes (3–9, 16, 19–23, 25, 29, 30, 33, 34). This group of enzymes contains large number of different catalytically active proteins, which are stored mainly in lysosomes and excreted either to the cytoplasm or to the extracellular compartment. The intracellular enzyme capacity is divided into bound and free factions. The bound fraction comprises all active forms of enzymes and proenzymes that are located inside of the endoplasmic reticulum, Golgi apparatus and lysosomes. Unlike those the free fraction contains only active form of enzymes present in cytoplasm and is not connected with any endoplasmic membranes (1, 3, 17).

Presently, the eucariotic endopeptidases – catehepsins – are among the most extensively studied groups of lysosomal enzymes. According to the classification of the International Union of Biochemical and Molecular Biology, which is based on mechanism of enzyme activity, structure of catalytic centre, optimum pH and sensitivity to inhibitors, cathepsins are divided into 4 primary groups: cysteine carboxypeptidases (EC 3.4.18) – cathepsin X; serine endopeptidase (EC 3.4.21) – cathepsin G; cysteine endopeptidase (EC 3.4.22) – cathepsin B, L, H, T, S, K, F, O and V; and aspartic endopeptidase (EC 3.4.23) – cathepsin D and E (9).

Special interest is given to cathepsin D (EC 3.4.23.5) which is involved in a variety of physiological processes such as proenzyme activation, enzyme inactivation, antigen presentation, hormone maturation, tissue remodelling, and bone matrix resorption (9, 11, 25, 33, 34). Elevated activity of this enzyme was also observed in many tumors including cancer of the breast, liver and prostate, as well as other pathological processes e.g. muscular dystrophy, hepatic cirrhosis, degenerative joint disease, and Alzheimer disease (2, 7–9, 15, 22, 23, 25, 29, 30, 34).

Experimental studies showed that expression of the cathepsin D gene, located on the short arm of the 11th chromosome (11p15.5) in human, is regulated by different steroid hormones, mostly estrogen and progesterone, and by growth factors like EGF, IGF-1 and TGF-α (2, 7, 23, 28, 32, 35). During the gen transcription the 1988 base long mRNA is synthesized, which in the course of translation produces pre-pro-cathepsin D of 412 amino acids (54 kDa). Post-translational modification, such as cutting off the N-terminal 20 amino acid fragment, glucosidation and phosphorylation, occurs in the endoplasmatic reticulum and later in the Golgi apparatus (26). This pre-cathepsin D is bound into the mannose-6-phosphate receptors and transferred to lysosomes, where due to acidic environment of this compartment the receptors split off and are excreted back to the Golgi apparatus (17). The succeeding modification such as cutting of the N-terminal 44 amino acid fragment and forth covalent modification form the catalytically active single and finally the mature double-chain cathepsin D molecule. The mature enzyme contains 339 amino acids (48 kDa) and is composed of the light (14 kDa) and heavy (34 kDa) chains. The quaternary structure is stabilised by a number of disulphide bonds (3, 9, 30).

In spite of the well-established and documented molecular aspects of cathepsin D physiology, its expression and distribution in several organs and tissues are still unclear.

The aim of the current study was to estimate the physiological activity and distribution of cathepsin D in mature rat liver using biochemical and immunohistochemical methods. The paper presents summary results of different experiments conducted previously for various scientific reasons. It was also important to evaluate the usefulness of the new, simple immunohistochemical Cathepsin D Kit for rat studies since only about its cross reactivity little information is available in the current literature.

Materials and Methods

The whole experiment was based on an animal experimental model, designed according to the general principles for animal experimentation (13) and the guidelines (#0038/2000) of the Local Bioethical Committee.

The experiment was conducted with outbred male Wistar having with initial body weights of 180±15 g. The animals were housed in standard laboratory plastic cages (maximum 5 rats per cage) at a room temperature of 20±3 °C in a daylight cycle. Standard laboratory chow LSM® (Wytwornia Pasz, Motycz – Poland) and filtered tap water were provided *ad libitum*. Food and water consumption were monitored daily.

The rats were individually identified by ear clipping done on the 5th day of the quarantine. After a two-week acclimation period, the animals were gathered in four groups, a minimum of 10 in a group. No xenobiotics were given to the animals through the entire experiment. The weight gain was monitored on days 0 and 4, then weekly until the day of the sacrification.

The animals were sacrificed consecutively on days 4, 42, 47 and 84 of the experiment, that corresponds to the 10th, 16th, 17th and 22nd week of postnatal age, respectively.

Animals were dissected immediately after decapitation. Internal organs were investigated during autopsy. The whole liver was removed. For immunohistochemical staining fragments of the liver were fixed in 10% buffered formaldehyde solution, processed routinely, embedded into paraffin blocks and sectioned of 4 µm slides. The Cathepsin D Kit (NCL-CathD-Paraffin, Novocastra Laboratories Ltd.; Newcastle, UK) with monoclonal mouse anti-human cathepsin D antibody (clone C5) was used according to the directions of the manufacturer. Epitope retrieval was applied with two cycles of heating in a microwave oven at 750 W for 5 minutes. The positive control was the sample of invasive ductal breast cancer, known to be strong by cathepsin D positive. The negative control was the section treated in the same way as in the study group, but with the omission of the primary antibody. The evolution of immunostaining was done by light microscope (Axioscop – Zeiss, Germany).

The remaining parts of the liver were frozen in liquid nitrogen and stored at –20 °C. Each liver, after being defrosted at the temperature of melting ice, was dissected, and a small part (about 1 g) of the right lobe was taken for biochemical investigation. The liver fragments were homogenised in 0.3 M sucrose at 4 °C in proportions of 1 g of

tissue to 5.0 ml of sucrose (Sigma Chemical Co., St. Louis, MO., USA). The homogenate was centrifuged at 2.200 g at 4 °C for 10 minutes. The supernatant was decanted and centrifuged at 35,000 g for 20 minutes. The supernatant contained the free fraction of enzyme. The precipitate was suspended in 5.0 ml of 0.3 M sucrose containing 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA), and stored for 24 hours at 4 °C. Triton was used to rupture the lysosomal membrane. After centrifugation at 35.000 g for 20 minutes, the supernatant contained the fraction of the bound enzyme.

Cathepsin D activity and protein level were assayed spectrophotometrically using substrates (Sigma Chemical Co., St. Louis, MO, USA) which form coloured complexes when reacted with the proteases. The whole method is described in detail elsewhere (5, 18, 21).

The obtained data were analyzed using STATISTICA 5.0 on a PC computer. The homogeneity of variances was examined using Kolmogoroff-Smirnoff test. Normal distribution data was analysed by Student's *t*-test. Inhomogeneous distribution data was analysed by Mann-Whitney U test. An $\alpha=0.05$ ($p<0.05$) was considered significant.

Results

There were no animal deaths, behavioural or any other clinical signs in the course of the experiment. No significant differences in food and water consumption or body weight gain were noted among the studied groups.

Immunohistochemical examination showed well stained Browicz-Kupffer cells (Kupffer cell) in several rat liver specimens in all experimental groups (Fig. 1). The staining pattern was cytoplasmic and granular. The positive reaction was observed in 7, 6, 5 and 6 livers obtained from rats sacrificed on days 4, 42, 47 and 84, respectively, of the study (Table I). However, a lack of immunohistochemical reaction was also seen in some of the livers. Occasionally, positive immunostaining of endothelial cells of central veins was also observed, even in livers with negative stained Browicz-Kupffer cells (Fig. 2, Table I). No activity of cathepsin D in hepatocytes was detected.

Statistical analysis showed insignificant differences in activity of the free and bound fraction of cathepsin D among the different age groups (Table I). However, the higher ($p>0.05$), age-related activity of the free fraction was observed in the youngest and in the two-middle groups of rats that were sacrificed on day 42 and 47 of experiment when compared with the oldest group. The bound fraction did not present such changes.

The positive immunostaining was found in livers with higher enzyme activity in biochemical investigation (Fig. 3). Significant elevation of the free and bound fractions was noted in positive cathepsin D immunostained livers ($n=25$) when compared with livers that lacked enzyme immunoexpression ($n=15$) (Table II).

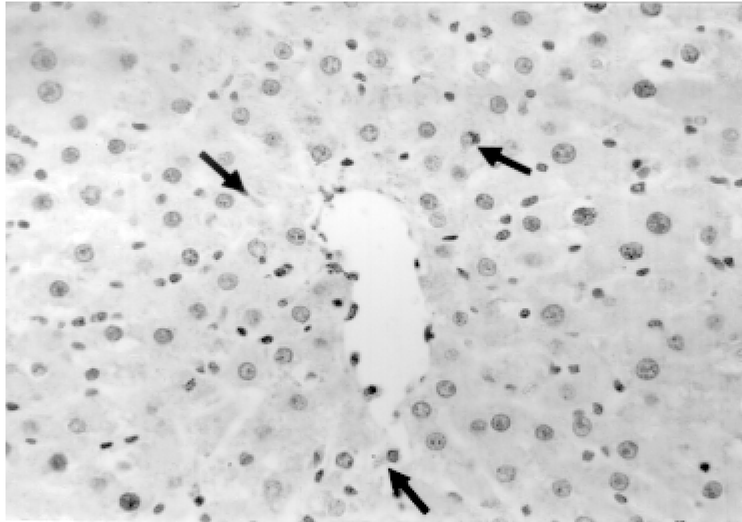

Fig. 1. Positive immunostaining for cathepsin D in Browicz-Kupffer cells (arrows) in rat liver (magn. ×200)

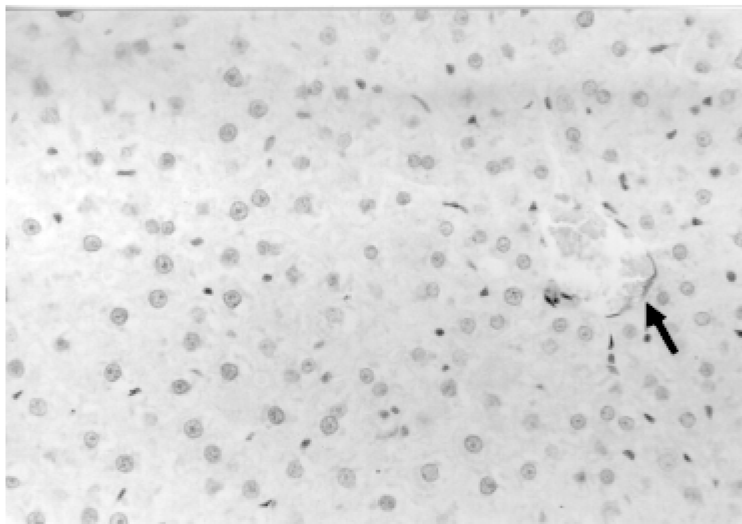

Fig. 2. Positive immunostaining for cathepsin D in endothelial cells of central vein (arrows) in rat liver with negative Browicz-Kupffer cell staining (magn. ×200)

Table I

Summary results of immunostaining and biochemical evaluation of the free (F) and bound (B) fractions of the hepatic cathepsin D in examined groups

Day of sacrification	Age (weeks)	N	Positive cell immunostaining		Cathepsin D activity (nmol/1g of protein)			
			Browicz-Kupffer	Endothelial	Fraction	Mean±SD	MIN	MAX
4	10	10	7	2	F	79.14±6.67	68.12	93.23
					B	67.43±5.86	57.03	74.45
42	16	10	6	-	F	78.02±10.73	64.55	98.45
					B	67.98±6.40	58.00	76.52
47	17	10	5	1	F	77.52±6.65	70.02	91.32
					B	67.49±4.42	62.00	73.03
84	22	10	6	1	F	76.14±7.83	68.44	90.45
					B	67.33±3.79	62.34	73.48

N – number of animals per group
SD – standard deviation
MIN – minimal value
MAX – maximal value
F – free fraction
B – bound fraction

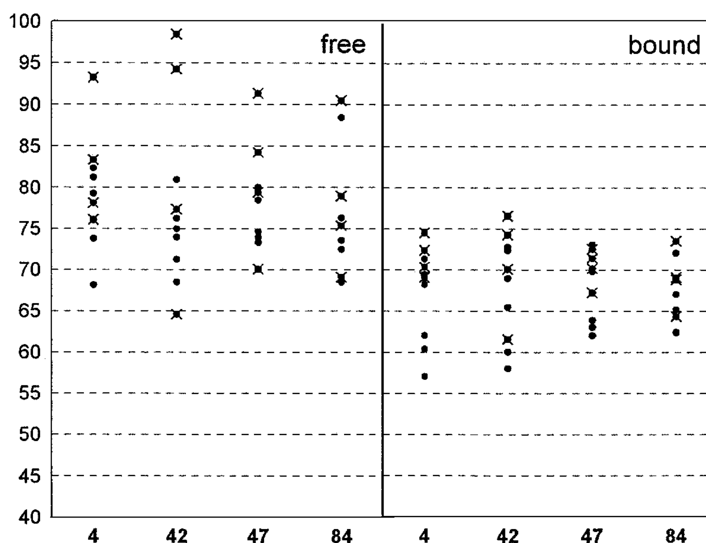

Fig. 3. Distribution of the activity of the free and bound fractions of hepatic cathepsin D (nmol/mg of protein) in day 4, 42, 47 and 84 of the experiment. The positive immunostaining are crossed with “X’s”

Table II

Activity of the free (F) and bound (B) fractions of cathepsin D in the positive and negative immunostained livers

Immunostaining	N	Cathepsin D activity (nmol/1g of protein)			
		fraction	Mean±SD	MIN	MAX
Positive	25	F	80.97±8.08*	64.55	98.45
		B	69.69±4.22*	58.00	76.52
Negative	15	F	72.25±3.30	68.12	78.45
		B	64.00±4.10	57.03	72.33

N – number of animals per group
SD – standard deviation
MIN – minimal value
MAX – maximal value
F – free fraction
B – bound fraction
* – $p<0.001$ when compared with the corresponding fraction from the negative immunostained liver group

Discussion

Our biochemical results showed insignificant differences of the liver cathepsin D activity among various age groups. Higher activity of the free fraction of the enzyme over the bound one was the only trend, and it was consequently observed in all the groups. Such results were seen previously in livers obtained from animals exposed to various xenobiotics like histamine H2 receptor antagonists, proton pump inhibitors and others, as well as intraperitoneally injected with physiological saline (4, 5). Similar profiles of free and bound fractions were also observed in rabbits and other experimental animals (3, 12, 21). Different control values observed among those studies are secondary to the different methodology used to determine enzyme activity and to species differences (3, 36). It should be noted that cathepsin D activity, in spite of the enzyme autolysis stability, is very labile since it could be elevated in direct tissue pathology, as well as in reaction process to diseases of the other organs (3, 6).

Different tissues and organs have their own specific enzyme activity, which vary among animal species. The differences were also noted between various groups of cells that form organs and even in various cell fractions. Similarly to our results, Knook (14) and Ansorge et al. (1) have reported that in the liver the highest total activity of cathepsin D is presented in Browicz-Kupffer cells. Their activity is at least three times higher than that in endothelial cells and about 5 to 24 times higher than the activity in hepatocytes. These results were later proved by Yokota et al. (37). Other studies done in rat liver and lung of guinea pig have shown that the lysosomal fraction has the highest intracellular cathepsin D activity (10, 12). About 50% lower activity was noted in

mitochondrial fraction. The cytoplasmic, microsomal and nuclear fractions presented lower enzyme activity. Their activity was higher in lung than in liver, which did not reach 10% of the lysosomal one.

Cathepsin D like other enzymes are heterogenic proteins. Different molecular forms have been detected already in various species. They differ in amino acid formula, molecular weight, spatial structure and isoelectric point. However, molecular mechanism of action and structure of the catalytic site is the same for all kinds and is formed by the present sequence Ile-Val-Asp-(Trp-Gly-Ther-Ser) (36). Different isoenzymes have been also obtained from single organs, e.g., 12 and 10 isoforms were detected in bovine uterus and spleen, respectively (27, 31). It was also proved that in pathological processes the new isoenzymes are synthesised. For example, cathepsin D obtained from the prostate cancer, unlike from the breast cancer, has a higher activity to release angiotensin from plasminogen (34). Similarly, the MCF-7 line of human hepatocellular cancer synthesizes cathepsin D with high contents of sialic acid and phosphate residues (8, 22).

Various molecular structures, especially amino acid contents, require other immunological properties that are the same for a given same species and different for heterogenic organisms. However, it spite of all the differences the monoclonal antibody against human cathepsin D seems to be useful for rat tissues examination, since similar staining, pattern and intensity was obtained in the positive human control tissue. Such cross-reactivity between rat and human was seen previously for various enzymes, receptors and other protein cell structures like receptors, filaments and regulated proteins (24).

It could be concluded that no differences in activity of hepatic free and bound fractions of cathepsin D were revealed in male Wistar rats of various reproductive age. The highest activity of cathepsin D was observed in rat Browicz-Kupffer cells when immunostained with the Cathepsin D Kit.

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REFERENCES

1. Ansorge S, Wideranders B, Riemann S, Brouwer A, Knook DL: Distribution of thiol-protein disulfide oxidoreductase, insulin-glucagon and cathepsin D in different cell types of the rat liver. Biomed. Biochim. Acta 43, 1213–1221 (1984)

2. Augereau P, Garcia M, Mattei MG, Cavailles V, Depadova F, Derocq D, Capony F, Ferrara P, Rochefort H: Cloning and sequencing of the 52K cathepsin D complementary deoxyribonucleic acid of MCF7 breast cancer cells and mapping on chromosome 11. Mol. Endocrinol. 2, 186–192 (1988)
3. Barrett AJ (1972): Lysosomal enzymes. In: Lysosomes. A Laboratory Handbook, eds. Dingle JH, North-Holland Publ. Co., Amsterdam, pp. 45–135.
4. Burdan F, Burak B, Burski K, Rucinski P, Pliszczynska M: Activity of cathepsins and morphological changes in the rat's liver after ranitidine and famotidine administration. Ann. Univ. Mariae Curie Sklodowska [Med], 54, 299–302 (1999)
5. Burdan F, Siezieniewska Z, Maciejewski R, Madej B, Radzikowska E, Wojtowicz Z: Hepatic lysosomal enzymes activity and liver morphology after short-time omeprazole administration. Exp. Toxic. Pathol. 53, 453–459 (2002)
6. Burdan F, Siezieniewska Z, Maciejewski Z, Burski K, Wojtowicz Z: Temporary elevation of pancreatic lysosomal enzymes, as a result of the omeprazole-induced paripancreatic inflammation in male Wistar rats. J. Physiol. Pharmacol. 51, 463–470 (2000)
7. Cavailles V, Augereau P, Rochefort H: Cathepsin D gene is controlled by a mixed promoter, and estrogens stimulate only TATA-dependent transcription in breast cancer cells. Proc. Natal. Acad. Sci. USA 90, 203–207 (1993)
8. Copony F, Rougeot C, Montcourrier P, Cavailles V, Salazar G, Rochefort H: Increased secretion, altered processing, and glycosylation of pro-cathepsin D in human mammary cancer cells. Cancer Res. 49, 3904–3909 (1989)
9. Cybulski M, Berbec H: The role of cathepsin D in cancer progression. Post. Bioch. 47, 224–231 (2001)
10. De Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F: Tissue fractionation studies. 6. Intracellular distribution of enzymes in rat-liver tissue. Bichem. J. 60, 604–617 (1955)
11. Deussing J, Roth W, Saftig P, Peters C, Ploegh HL, Villadangos JA: Cathepsin B and D are dispensable for major histocompatibility complex class II-mediated antigen presentation. Proc. Natl. Asoc. Sci. USA 98, 4516–4521 (1998)
12. Ferren LG, Strauber WT, Kalnitsky G: Differential centrifugation studies of guinea pig lung protease. Proc. Soc. Exp. Biol. Med. 159, 239–244 (1978)
13. Gorska P: Principles in laboratory animals research for experimental purpose. Med. Sci. Monit. 6, 171–180 (2000)
14. Knook DL: The role of lysosomal enzymes in protein degradation in different types of rat liver cells. Acta Biol. Med. Chem. 36, 1747–1752 (1977)
15. Kute TE, Grondahl-Hansen J, Shao SM, Long R, Russell G, Brunner N: Low cathepsin D and low plasminogen activator type 1 inhibitor in tumor cytosoles defines up of node negative breast cancer patients with low risk of recurrence. Breast Cancer Res. Treat. 47, 9–16 (1998)
16. Lah TT, Kos J: Cysteine proteinases in cancer progression and their clinical relevance for prognosis. Biol. Chem. 379, 125–130 (1998)
17. Lemansky P, Gieselmann V, Hasilik A, von Figura K: Cathepsin D and beta-hexosaminidase synthesized in the presence of 1-deoxynojirimycin accumulate in the endoplasmic reticulum. J. Biol. Chem. 259, 10129–10135 (1984)
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951)
19. Maciejewski R, Burdan F, Hermanowicz-Dryka T, Wojcik K, Wojtowicz Z: Changes in the activity of some lysosomal enzymes and in the fine structure of submandibular gland due to experimental diabetes. Acta Physiol. Hung. 86, 127–137 (1999)
20. Maciejewski R, Burski K, Baj J, Madej B, Burdan F, Dabrowski A, Brakowiecki F: Changes in pancreatic lysosomal enzymes activity as the potential factors leading to diabetic enteropathy. J. Physiol. Pharmacol. 52, 823–834 (2001)
21. Maciejewski R, Kaus M, Wojtowicz Z, Burdan F, Burski K: Changes in the activity of pancreatic cathepsin D, L and acid phosphatase in the course of alloxan-induced diabetes mellitus in rabbits. Med. Sci. Res. 28, 113–116 (2000)

22. Maguchi S, Taniguchi N, Makita A: Elevated activity and increased mannose-6-phosphate in the carbohydrate moiety of cathepsin D from human hepatoma. Cancer Res. 48, 362–367 (1988)
23. Maudelonde T, Martinez P, Brouillet JP, Laffargue F, Pages A, Rochefort H: Cathepsin-D in human endometrium: induction by progesterone and potential value as a tumor marker. J. Clin. Endocrinol. Metab. 70, 115–121 (1990)
24. Novocastra Laboratories. Table of cross-reactivities. Novocastra Laboratories Ltd, Newcastle upon Tyne. 2002.
25. Olszewska D, Drewa T, Markiewicz R, Drewa J, Wozniak A, Maciak R: The significance of cathepsin B and D in the physiological and pathological processes. Pol. Merkuriusz Lek. 10, 65–70 (2001)
26. Paris A, Strukey B, Pungercar J, Renko M, Dolenc I, Turk V: Molecular cloning and sequence analysis of human preprocathepsin C. FEBS Lett. 369, 326–330 (1995)
27. Press E, Porter RR, Cebra J: Isolation and properities of a proteolytic enzyme, cathepsin D, from bovine spleen. Biochem. J. 74, 501–514 (1960)
28. Redecker B, Heckendorf B, Grosch HW, Mersmann G, Hasilik A: Molecular organization of the human cathepsin D gene. DNA Cell Biol. 10, 423–431 (1991)
29. Rochefort H: Cathepsin D in breast cancer: mechanisms and clinical applications, a 1999 overview. Clin. Chim. Acta 291, 157–170 (2000)
30. Rochefort H, Liaudet-Coopman E: Cathepsin D in cancer metastasis: a protease and a ligand. APMIS 107, 86–95 (1999)
31. Sapolsky A, Woessner JF Jr: Multiple forms of cathepsin D from bovine uterus. J. Biol. Chem. 247, 2069–2076 (1974)
32. Sheikh MS, Augereau P, Chalbos D, Garcia M, Rochefort H: Retinoid regulation of human cathepsin D gene expression. J. Steroid. Biochem. Mol. Biol. 57, 283–291 (1996)
33. Soderstrom M, Salminen H, Glumoff V, Kirschke H, Aro H, Vuorio E: Cathepsin expression during skeletal development. Biochim. Biophys. Acta 1446, 35–46 (1999)
34. Tsukuba T, Okamoto K, Yasuda Y, Morikawa W, Nakanishi H, Yamamoto K: New functional aspects of cathepsin D and cathepsin E. Mol. Cells. 31, 601–611 (2000)
35. Wang F, Duan R, Chirgwin J, Safe SH: Transcriptional activation of cathepsin D gene expression by growth factors. J. Mol. Endocrinol. 24, 193–202 (2000)
36. Worowski K, Ostrowska H: Cathepsin D. Post. Biol. Kom. 7, 119–147 (1980)
37. Yokota S, Tsuji H, Kato K: Localization of cathepsin D in rat liver. Immunocytochemical study using post-embedding immunoenzyme and protein A-gold techniques. Histochemistry 82, 141–148 (1985)