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Review article

Quantitative determination and localization of cathepsin D and its inhibitors

Alina Minarowska[1], Alicja Karwowska[2], Marek Gacko[3]

[1]Department of Clinical Nursing, Medical University of Bialystok
[2]Department of Epidemiology and Hygiene, Medical University of Bialystok
[3]Department of Vascular Surgery, Medical University of Bialystok

Abstract: A literature survey was performed of the methods of quantitative assessment of the activity and concentration of cathepsin D and its inhibitors. Usefulness of non-modified and modified proteins and synthetic peptides as measurement substrates was evaluated. The survey includes also chemical and immunochemical methods used to determine the distribution of cathepsin D and its inhibitors in cells and tissues.

Key words: cathepsin D, cathepsin D inhibitors, activity, concentration, cell distribution, tissue distribution

Cathepsin D (EC 3.4.23.5) is a lysosomal aspartyl endopeptidase, localized in all cells and tissues, except for mature erythrocytes [1-3]. Methods used to determine the activity, concentration and cellular distribution of cathepsin D, but not its inhibitors, have previously been the subject of literature reports [4-12]. However, since the time of their publication a number of new substrates and analytical techniques have been implemented.

Structure, specificity, mechanism of action

Cathepsin D is synthesized in the rough endoplasmic reticulum as preprocathepsin D, built up of 412 amino acid residues [13-15]. As a result of cleavage of the 20-amino acid signal prepeptide, it is converted into procathepsin D which undergoes glycosylation and disulphide bridges are formed in its molecule. Procathepsin D is transported from cisterns of the rough endoplasmic reticulum to the Golgi apparatus, from which, with the involvement of mannoso-6-phosphate (M-P-6) receptors, it is transferred to primary lysosomes [16,17]. As the M-6-P receptors are known to occur in the primary lysosomes but not in the mature ones, they can be used to distinguish between these two types of lysosomes [18]. In the acidic environment of the lysosomes (pH 4.5-5.5), due to autocatalytic cleavage of the 44-amino acid propeptide from the N-terminal molecule, procathepsin D is converted into the active one-chain form. The actions of cysteine proteinase, aminopeptidases and carboxypeptidases lead to the formation of an active two-chain form of cathepsin D (Fig. 1). These chains are bound by hydrophobic bonds. The molecular weight of the ultimate mature form of cathepsin D is 48 (14+34) kDa. The proteolytic activities of the one-chain and two-chain forms are very similar [19,20]. Modification of the polypeptide chain, different oligosaccharide composition types and phosphorylation/dephosphorylation in the amino saccharide residues contribute to marked molecular heterogenicity of cathepsin D and cause differences in isoelectric points of the respective isoenzymes between pH 4.5 – 6.5 [21,22].

The use of peptides with the known primary structure allows identification of amino acid residues that form peptide bonds cleaved by cathepsin D. For this purpose, synthetic peptides [23] and chains A and B of bovine insulin can be used (Fig. 2). Cathepsin D cleaves the peptide bonds found within the polypeptide chain, formed by carboxyl groups of the hydrophobic amino acid residues: aromatic – trypto-

Correspondence: A. Minarowska, Dept. of Clinical Nursing, Collegium Novum, Waszyngtona 15A Str., 15-274 Białystok, Poland; tel./fax: (+4885) 7468581, e-mail: minar@mp.pl

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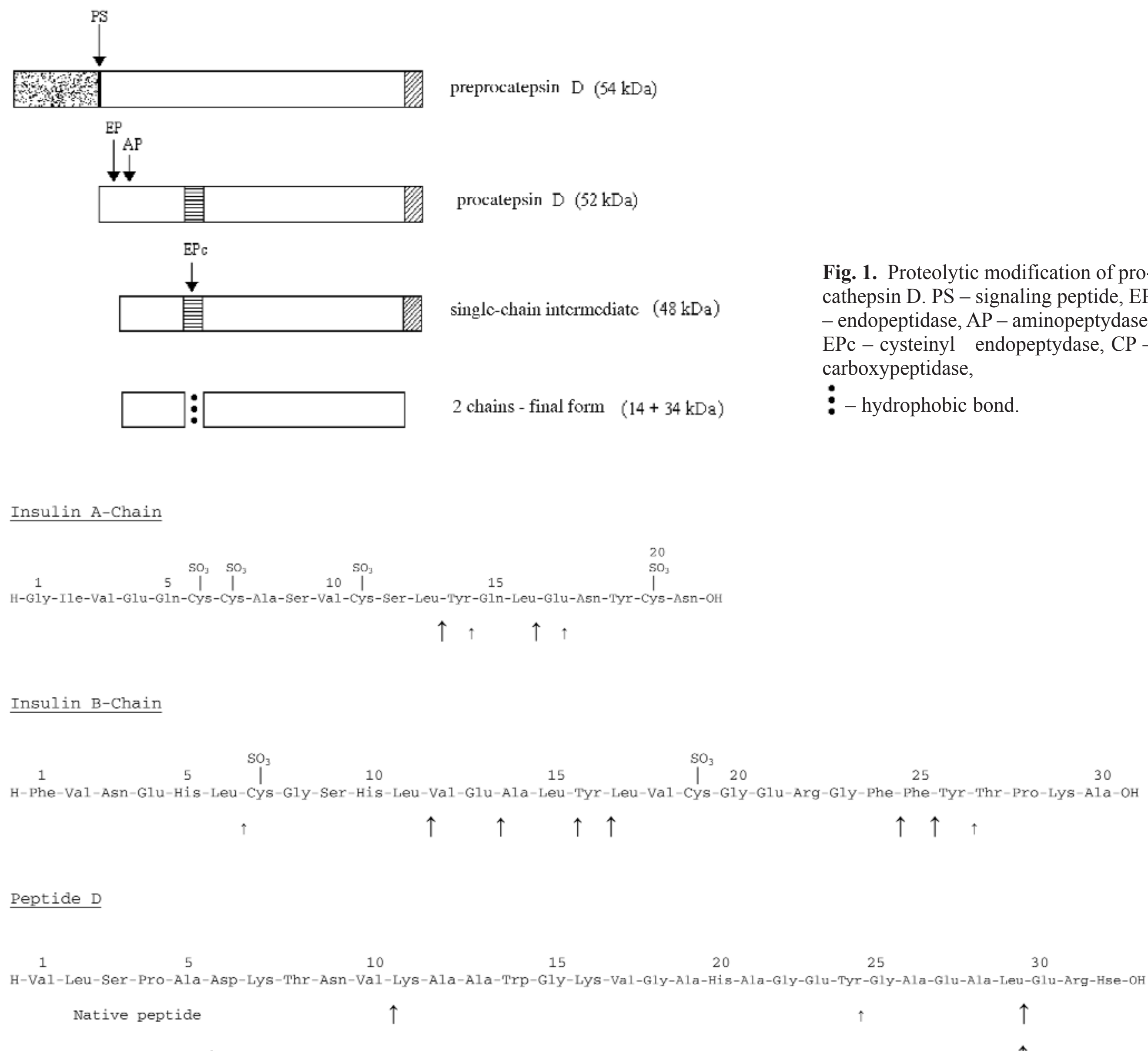

Fig. 1. Proteolytic modification of procathepsin D. PS – signaling peptide, EP – endopeptidase, AP – aminopeptydase, EPc – cysteinyl endopeptydase, CP – carboxypeptidase,

⁞ – hydrophobic bond.

Fig. 2. Specificity of porcine spleen cathepsin D. The specificity of the enzyme is shown in the sites of cleavage in four peptides; oxidized A chain of bovine insulin , oxidized B chain of bovine insulin, peptide D, and acetylated peptide D. The vertical arrows below the sequences indicate the relative preference of the hydrolyzed site. They are assigned either from the peptide yields or from the carboxypeptidase A digestions [24]. ↑ – high yield sites; ↑ – medium yield sites; ↑ – low yield sites.

phan, tyrosine and phenylalanine, and long-chain aliphatic amino acids – leucine and isoleucine (Fig. 3) [23].

Two aspartic acid residues, *i.e.* Asp33 and Asp231 constitute the catalytic site of cathepsin D. Tyr205 and to a lesser extent other amino acid residues evolve as the third component of the catalytic triad. The course of cathepsin D-catalyzed hydrolysis of the peptide bond is presented in Fig. 4. The hydrolysis starts with two simultaneous proton transfers (reaction a), one between the water molecule and the carboxyl ion Asp33, the other between the carboxyl group of Asp231 and the oxygen atom of the carboxyl group in the substrate. These transfers facilitate formation of indirect tetraedric product. An analogous mechanism of double transfer leads to decomposition of the indirect product (reaction b), in which a proton of the hydroxyl group is transferred onto the Asp33, whereas the Asp231 is transported onto the nitrogen atom, and thereby the peptide bond -CO-NH- is hydrolyzed in the substrate. The first product of reaction with the free carboxyl group (R_1-COOH) and the second product of reaction with the free amino group (R_2-NH_2) are generated, and cathepsin D is released (reaction c).

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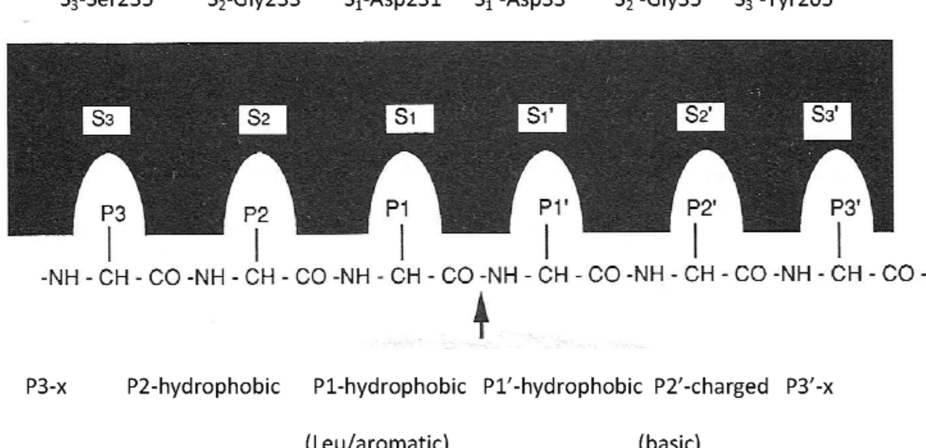

Fig. 3. The Schecter and Berger nomenclature for binding of peptide substrate to cathepsin D. The cathepsin D is represent as the shaded area. P1-P1' are side chains of six amino acids, and S1-S1' are the corresponding subsides on the cathepsin D.

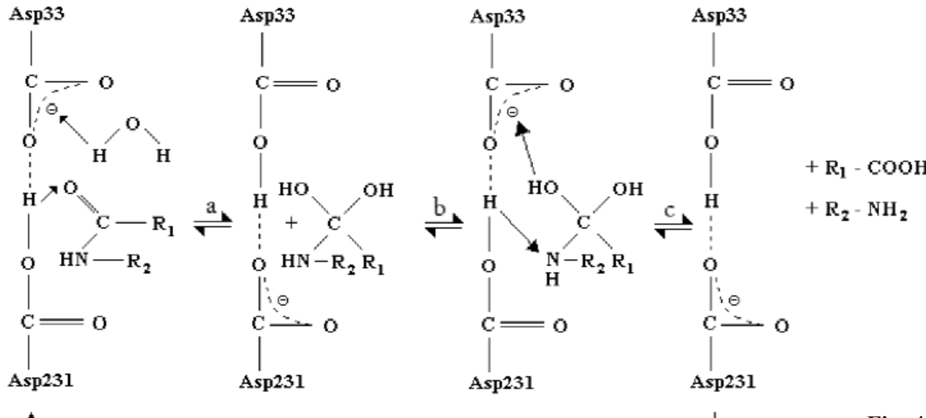

Fig. 4. Hydrolysis of a peptide bond [25].

Cathepsin D cleaves only certain peptide bonds in proteins, and does it in a defined sequence. Rupture of even one peptide bond in a native protein molecule changes its spatial structure. The glomerular structure of native protein is converted into the fibril-like one, thus rendering peptide bonds accessible to cathepsin D and leading to progressing degradation of protein molecule. In this process, the mass concentration of the protein remains stable, whereas the molar concentration increases. Progressing fragmentation, spatial changes in the protein and effects exerted on the peptide fragments of aminopeptidases and carboxypeptidases prolong protein degradation until amino acids are formed.

The optimum pH and range of cathepsin D activity

Cathepsin D acts in an acidic pH range (3.0 – 4.5). The optimum pH depends on the type of protein and synthetic peptide [26,27], as well as whether cathepsin D occurs free or in bonds. Cathepsin D binding to the lysosomal membrane or a constant carrier increases the pH optimum [28]. The optimum also depends on the structure of protein molecule. Denaturated proteins undergo cathepsin D hydrolysis at a higher pH as compared to native proteins. Moreover, denaturation frequently increases protein susceptibility to the action of cathepsin D.

Complexification of hemoglobin with haptoglobin reduces the optimum pH from 3.5 to 2.8 for degradation and simultaneously causes a 50% decrease in degradation rate 50% (Fig. 5) [29].

Material preparation, conditions and specificity of assays

For diagnostics purposes, the activity and concentration of cathepsin D are determined in blood plasma, secretions, body fluids, excretions, tissue homogenates and in isolated lysosomes [31-34].

Plasma is obtained by centrifugation of blood collected to 3.8% sodium citrate (9:1 v/v ratio). Blood serum is not useful for cathepsin D activity determination. Plasma activity of cathepsin D is found to be lower than its serum activity [35]. Higher serum activity of cathepsin D indicates its release from platelets

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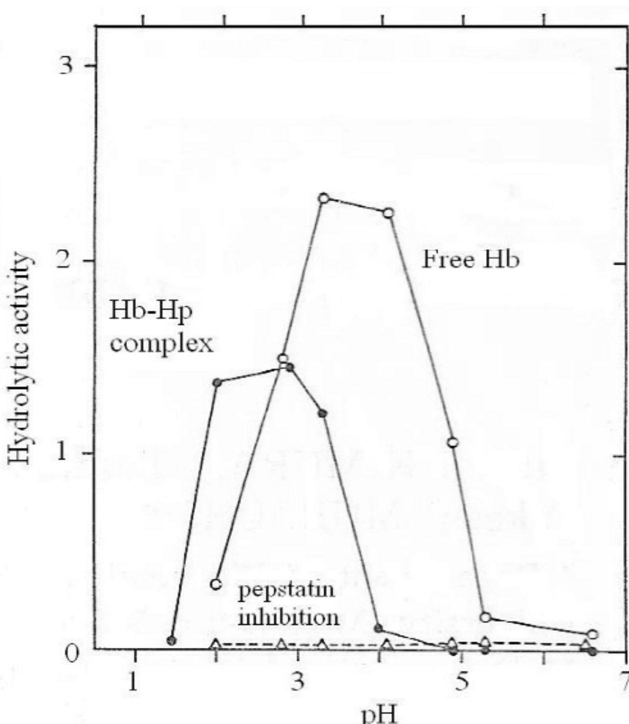

Fig. 5. pH profiles of proteolyses by cathepsins. The ordinate represents the hydrolytic activity in arbitrary units. ○, free Hb; •, Hb-Hp complex; Δ, pepstatin inhibitor for both [30].

that occurs after blood collection [35]. The amount of the released enzyme depends on platelet count and susceptibility of these cells to the release. Therefore, for diagnostic purposes, cathepsin should be determined in blood plasma. Cells from secretions, body fluids and excretions are removed by centrifugation directly after collection, prior to measurement of cathepsin D activity. Preparation of cell and tissue material for this measurement consists in homogenization and fractionation of homogenate by centrifugation/ultracentrifugation. In cells and tissues, cathepsin D activity is assessed in the whole homogenate (total activity), cytosole (free activity) and in lysosomes (bound activity). The activity of cathepsin D lysosomal fraction can only be roughly estimated due to heterogeneity of lysosomes and their partial sedimentation with other organelles [36]. The whole homogenate is prepared in 0.15 mol/l KCl using a flow homogenizer, in which tissue passes only once and at a definite time through the working area of the cutting blade or knife homogenizer [37,38]. Other methods used to prepare the whole homogenate include pulverization under liquid nitrogen, using a hand or mechanical [39,40] pulverizer. The homogenate, irrespective of the mode of preparation employed, is filtered through nylon cloth (pore size 0.12-0.15 nm) [37,41]. The activity of cathepsin D is determined in a noncentrifuged filtrate. Centrifugation causes sedimentation of approximately 20-30% of cathepsin D activity with fragments of lysosomal and

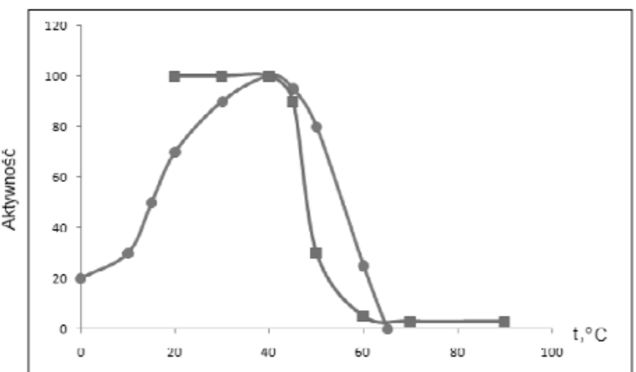

Fig. 6. Activity (●) and stability (■) of cathepsin D as a function of temperature [57].

cell organelle membranes [42,43]. In order to obtain cytosole and lysosomes, homogenate is prepared in 0.25 mol/l saccharose, using a Potter-Elvehjem homogenizer with smooth walls of a glass cylinder and a teflon piston, between which there is a 0.22 mm gap [44]. Cytosole is separated from lysosomes through homogenate ultracentrifugation [45,46] or precipitation of lysosomes at pH 5.0 [47]. The soluble cathepsin D fraction found in the solution constitutes supra-sediment after ultracentrifugation at 100000 x g, for one hour. Noncovalent interactions (ionic, hydrophobic, Me^{2+} cation bridges) condition cathepsin binding to the membranes. Due to substantial durability of lysosomes, after a 48h storage in an isotonic environment at a temp. of 0-2°C, only 5-10% of cathepsin D passes to the environment. The material submitted for determination is stored in 10% DMSO or 25% glycerol at a temp. of -20°C or in a frozen state, in a freezer (temp. -75°C) or in a liquid nitrogen (temp. -182°C) [48,49].

Procathepsin D, at an acidic pH, undergoes rapid autoactivation. Its active form does not require activators and does not possess endogenous cell inhibitors. As plasma α2-macroglobulin does not suppress the action of cathepsin D at an acidic pH, total activity of this proteinase in plasma is determined *in vitro*. The *in vivo* activation and action of cathepsin D is conditioned by the acidic environment of lysosomes (pH 4.5-5.5). Interlysosomal pH is measured using one of the methods described [50-52]. The propeptyde cleaved in this process is reported to have a certain regulatory role in procathepsin D activation [53,54].

The most common substrate used to determine cathepsin D activity is a 6% HCL-denaturated globin with urea [55] or the one obtained after treatment of hemoglobin with HCL-acidified acetone [56]. Hemoglobin digestion by cathepsin D takes place at 37°C. Elevation of the reaction temperature to 40-45°C causes only a slight increase in the reaction products. At higher temperatures, the enzyme becomes inactivated (Fig. 6). The incubation time determines the increase

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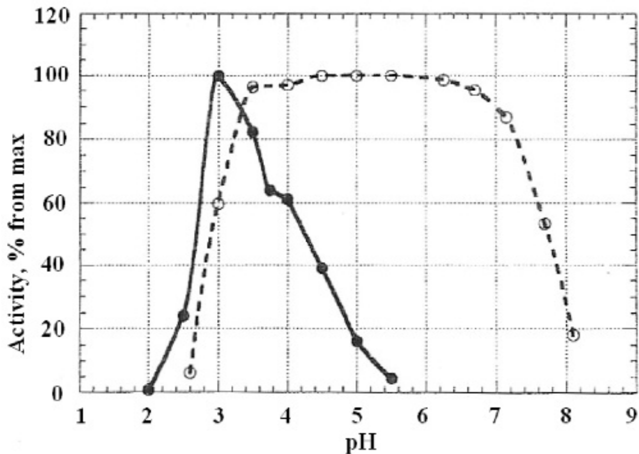

Fig. 7. pH dependance of cathepsin D conformation and activity in solution. Enzyme activity (solid line) and enzyme stability (dash line) of cathepsin D as a function of pH [61].

in degradation products within the absorbance range of 0.2 to 0.6. In low-activity material, the sample volume should be relatively large whereas the volume of hemoglobin with a final concentration of 1-2% – relatively small [11]. A cathepsin D sample of 200-500 ng is sufficient for a single determination [49]. When the activity is very low, the sample should be densified using immunoprecipitaion technique [58] or by one of the standard methods [59]. The pH optimum of cathepsin D activity depends on the type of substrate and the composition and ionic strength of a buffer. The buffers with the ionic strength ranging between 0.01 do 0.1 mol/l are used: glycin buffer (pH 2.2 – 3.0), formate buffer (pH 2.6 – 4.8), acetate buffer (pH 3.6 – 5.6), and universal Britton-Robinson buffer (pH 1.81 – 11.94) [60]. The Britton-Robinson buffer with various pH values is supplemented with appropriate amounts of NaCl to ensure the same ionic strength [60]. The correlation between cathepsin and pH, as well as stability of this proteinase at various pH values are shown in Fig. 7.

Determination of activity and concentration

The quantitative assessment of cathepsin D is based on its catalytic properties (activity measurement) and antigenic properties (concentration measurement). In the activity measurement, the number of active molecules is determined, whereas in the concentration measurement the total number of all molecules, both active and inactive, is estimated. Since procathepsin D does not need activators and does not have endogenous cell inhibitors, its total activity can be determined in tissue material. The actions of other endopeptidases and exopeptidases found in the non-fractionated material are eradicated by their inhibitors (Table 1). Pepstatin, the inhibitor of cathepsin D and other aspartyl proteinases, does not inhibit the activities of cystein cathepsins, seryl cathepsins or metaloproteases, and inhibitors of these cathepsins do not suppress cathepsin D action. A properly-selected set of inhibitors allows differentiation of cathepsin D from cathepsin E, pepsin and rennin, which are also aspartyl proteinases (Table 2).

Table 1. Inhibitors of different proteinase classes.

Inhibitor, concentration	Proteinase class
Pepstatyna, 1 μmol/l DAN + Cu^{2+}, 1 mmol/l	Aspartyl
PCMB, 1 mmol/l E-64, 10 μmol/l	Cysteinyl
PMSF, 1 mmol/l 3, 4-DCI, 0,1 mmol/l	Serylowe
1,10-fenanrtolina, 1 mmol/l EDTA, 2 mmol/l	Metalloprotease
Inhibitors coctail E-64, 0,4 mmol/l 3,4-DCI, 2 mmol/l 1,10-fenantrolina, 2 mmol/l	Cysteinyl Seryl Metalloprotease

E-64 – L-3-carboxy-2,3-trans-epoxy-propionyl-leucylamido/guanidyne/buthane; 3,4-DCI – 3,4-Dichloroisocoumarin (3,4-Dichloro-2-benzopyran-1-one).

Table 2. Vulnerability of human aspartyl proteases to inhibitors; + inhibitory effect, - lack of inhibition [62].

Inhibitor	Cathepsin D	Cathepsin E	Pepsin	Renin
Pepstatin A	+	+	+	+
Ascaris sp. derived inhibitor	-	+	+	-
Antibodies against cathepsin D	+	-	-	-

Cathepsin D cleaves only certain peptide bonds in proteins and does it in a defined sequence. In this process, mass concentration remains stable whereas molar concentration of its fragments increases. The activity of cathepsin D is most frequently manifested by the amount of generated degradation products. More seldom, its activity is shown as the number of microequivalents of cleaved peptide bonds per time unit.

Native, denaturated and labeled proteins

Protein susceptibility to the action of cathepsin D is determined by the composition and sequence of amino acid residues in the polypeptide chain and by its spatial structure. Hence, different susceptibility of various proteins to the action of this proteinase (Table 3).

The α- and β-globins which build the hemoglobin molecule are most prone to the action of cathepsin D.

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Table 3. Degradation of different proteins by cathepsin D in different pH [57].

Protein	pH		
	3,0	3,5	5,0
Hemoglobin	100	90	30
α-globulin	12	12	8
β-globulin	10	10	5
γ-gloglobulinbulina	0	0	0
Albumin	15	12	5
Fibrynogen	0	0	0
Fibrine	12	8	8
Casein	15	20	12

The hemoglobin molecule contains two α- chains and two β-globin chains forming dimers composed of one chain α and one chain β. The α chain included in bovine hemoglobin molecule is built up of 141 whereas β chain contains 146 amino acid residues. Their amino acid sequence and the cleavage site are presented in Fig. 8. At the junctions of the homonymous subunits (α-α, β-β), polar amino acid residues are found to predominate, whereas the heteronymous subunits show the predominance of (α-β) hydrophobic residues. Hemoglobin tetramer is stabilized mainly due to hydrophobic interactions between the heteronymous subunits. Each α- and β-globin chain contains one hem and one ferrous ion (Fe^{2+}). Hem accounts for 4% and iron for 0.34% of the hemoglobin molecule. The polypeptide α- and β-globin chains are in 80% constituted by the α-helis structure and in 20% by a disordered structure [63,64]. The fact that bovine

A

Hemoglobin α-chain

Cleaved	Bovine	Human	Canine
1	V*LSPA	V*LSPA	V*LSPA
24	AAEY*GAEA	AGEY*GAEA	ADGY*GGEA
32	LERM*FLSF	LERM*FLSF	LDRM*FOSF
109	LLVT*LASH	LLVT*LAAH	LLVT*LACH
134	ANVS*TVLT	ASVS*TVLT	AAVS*TVLT
137	STVL*TSKY	STVL*TSKY	STVL*TSKY

Hemoglobin β-chain

Cleaved	Bovine	Human	Canine
6	TAEE*KAAV	TPEE*KSAS	TAEE*KSLV
14	VTAF*WGKD	VTAL*WEKV	VSGL*WGKV
30	LGRL*LVVY	LGRL*LLVV	LGRL*LIVY
31	GRLL*VVYP	GRLL*VVYP	GRLL*IVYP
40	TQRF*FESF	TQRF*FESF	TQRF*FDSF
44	FESF*GDLS	FESF*GDLS	FDSF*GDLS
53	ADAV*MNNF	PDAV*MGNP	PDAV*MSNA

B

Hemoglobin α-chain

Cleaved	Bovine	Human	Canine
29	AEAL*ERMF	AEAL*ERMF	GEAL*DRTF
33	ERMF*LSFP	ERMF*LSFP	DRTF*OSFP
36	FLSF*PTTK	FLSF*PTTK	FOSF*PTTK
45	YFPH*FDLS	YFPH*FDLS	YFPH*FDLS
109	LLVT*LASH	LLVT*LAAH	LLVT*LACH
110	LVTL*ASHL	LVTL*AAHL	LVTL*ACHH
129	LDKF*LANV	LDKF*LASV	LDKF*LAAV

Hemoglobin β-chain

Cleaved	Bovine	Human	Canine
6	TAEE*KAAV	TPEE*KASV	TAEE*KSLV
14	VTAF*WGKD	VTAL*WEKV	VSGL*WGKV
30	LGRL*LVVY	LGRL*LLVY	LGRL*LIVY
31	GRLL*VVYP	GRLL*VVYP	GRLL*IVYP
40	TQRF*FESF	TQRF*FESF	TQRF*FDSF
44	FESF*GDLS	FESF*GDLS	FDSF*GDLS
129	LQAD*FQKV	VQAA*YQKV	VQAA*YQKV

Fig. 8. (**A**) Human cathepsin D – known cleavage sites in bovine hemoglobin are shown alongsite orthologous sites in human and canine hemoglobin. (**B**) Schistosoma japonicum cathepsin D – known cleavage sites in human hemoglobin are shown alongsite equivalent sites in bovine and canine hemoglobin [65].

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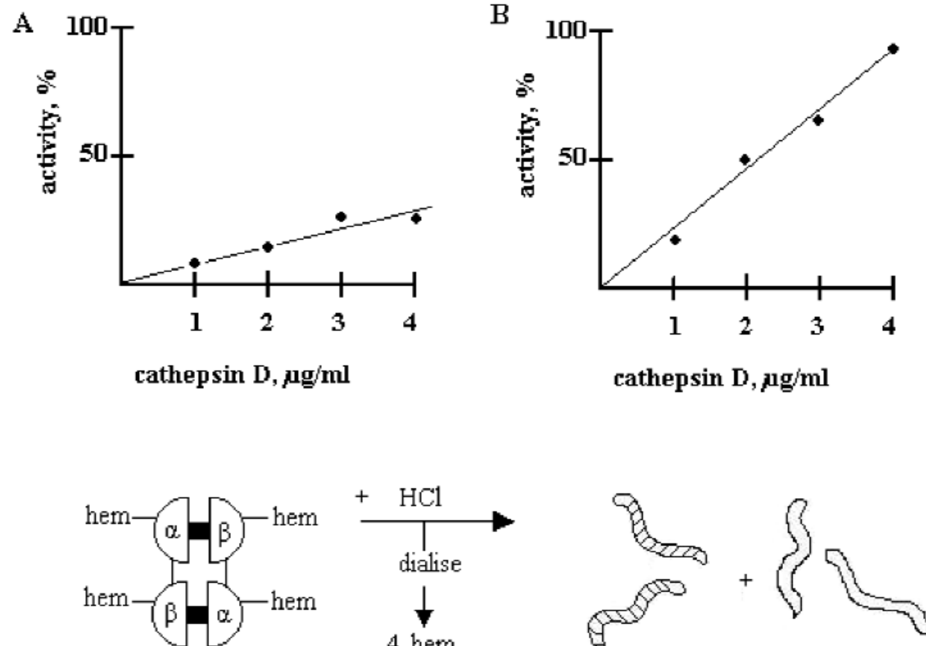

Fig. 9. Denaturation of hemoglobin by HCl and its influence on globin degradation by cathepsin D. (**A**) – naive hemoglobin; (**B**) – α and β denaturated globin.

α- and β-globin chains do not possess disulphide bridges that would reduce the action of the proteinase is their advantage.

Mixtures of α-globin and β-globin obtained by hemoglobin denaturation with hydrochloric acid, hydrochloric acid and urea, sodium hydrogen, sodium hydrogen and urea are most frequently used to determine cathepsin D activity [66]. Due to their action, Hem is detached, bonds that join α- and β-globin chains torn apart, their structure undergoes denaturation and becomes more susceptible to cathepsin D action (Fig. 9). The mixture of α- and β-globin, obtained in that way is traditionally referred to as 'hemoglobin'. High solubility in a wide pH range (1.0-12.0) is another advantage of α- and β-globins, allowing them to be used for the determination of pH optima for cathepsin D activity. The globin concentration used to determine the activity of cathepsin D ranges from 0.5 to 5%. Denaturation with the involvement of urea increases at the same time globin solubility in a wide pH range and the number of non-precipitated TCA products of its degradation [67,68].

Similarly, hemoglobin treatment with HCL-acidified acetone causes hem detachment, disruption of connections between globins and their denaturation [56,71-73]. Simultaneously, globin molecules undergo precipitation and are separated from hem dissolved in acetone by centrifugation.

Globin hydrolysis termination is accomplished using trichloroacetate acid (TCA) that precipitates undecomposed globin. The final concentration of TCA accounts for 0.1 – 0.8 mol/l (2.5 – 20.0%). Depending on TCA concentration, products with various molecular masses are precipitated. Application of higher TCA concentrations facilitates separation of a thick precipitate by filtration or centrifugation, but reduces the number of products left in the solution. Degradation products soluble in TCA are separated from the precipitated protein by filtration or centrifugation. In a clear filtrate/suprasediment, the concentration of hydrolysis products is determined by direct measurement of absorbance at 280 nm [5] or fluorescence [74,75], dependent on tyrosyl and tryptophanyl residues. However, direct measurement of absorbance at 280 nm in nonpurified material is loaded with error. Nucleic acids contained in such a material are degraded by nucleases and released nucleotides increase the measurement values [76,77]. Degradation products are more frequently determined following addition of staining reagent, via absorbance measurement at a suitable wavelength. The reagents used for staining of degradation products include copper reagent (microbiuretic) and Folin and Ciocalteau's reagent applied separately or simultaneously, ninhydrin reagent, 2,4,6-trinitrobenzenosulphic acid and o-ftalaldehyde [78-81].

Hemoglobin denaturation and assessment of cathepsin D activity described below is recommended for routine assays.

A. Hemoglobin denaturation: hemoglobin (6g) is suspended in 35 ml of distilled water, with addition of 15 ml 1 mol/l HCl and then incubation is carried out at 37°C for 30 minutes. The pH is elevated up to 3.5 by means of 1 mol/l NaOH, next distilled water is added to 90 ml and 10 ml of 2.0 mol/l acetate buffer (pH 3.5); a 6% solution of globin in acetate buffer, pH 3.5, is obtained in this way.

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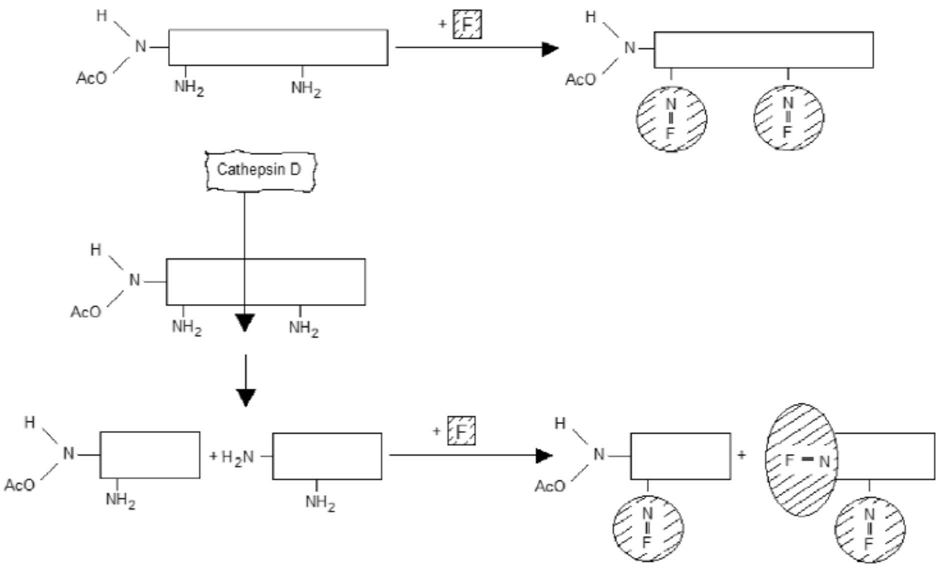

Fig. 10. Scheme of reaction of fluorescamine with proteins as the basis for the development of a metod for the measurment of proteolytic degradation. The stripped area represents fluorescence and its relative size stands for the relative fluorescence of the N-terminal and ?-amino groups after reaction with fluorescamine [89].

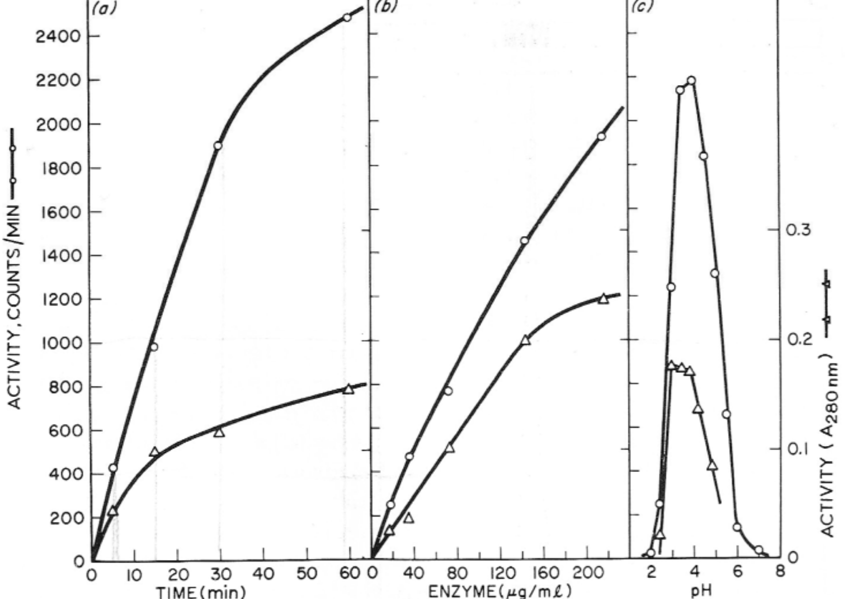

Fig. 11. Reaction of cathepsin D on methyl-^{14}C-glycinated hemoglobin (o–o) and acid-denaturated hemoglobin (Δ–Δ) as a function of (**a**) time, (**b**) protein concentration and (**c**) pH [91].

B. Assay: 0.1 ml of 6% globin (pH 3.5) is added to 0.4 ml sample (homogenate, plasma, the same pH) and incubation is performed at 37°C for 1-6 hours (depending on activation). The reaction is discontinued by addition of 0.5 ml of 5% TCA acid containing 2.5 mol/l urea. The sample in which TCA acid was added at time zero is referred to as 'control'. All samples undergo centrifugation (1500 x g, 2°C, 30 minut). The amount of 0.25 ml of suprasediment fluid is supplemented with 1.5 ml of copper reagent (1 volume of 0.5% $CuSO_4$ x H_2O in 1% sodium citrate x 5 H_2O and 30 volumes of 10% sodium carbonate). After 10 minutes, 0.25 ml of Folin and Ciocialteau's reagent diluted with distilled water (2:1 v/v ratio) is added and after further 30 minutes, absorbance is measured at 750 nm. Results are read from a calibration curve designed according to standard tyrosine solutions (10-200 nmol/ml).

The use of labeled hemoglobin/globin considerably simplifies the methods applied to determine the activity of cathepsin D. As a result of fragmentation of a globin molecule, the molecule bound marker after addition of trichloroacetate acid remains in the solution, its concentration reflecting the molecule activity. The globin labeled with chromophores, fluorophores and radioelements are used [3,82]. The chromophore consists of the nitric group (nitro-globin) and azo group (azo-globin). Fluorophores include fluorescamine, fluorescein isothiocyanate, rhodamine B isothiocyanian, 1-amino-8-naphtalene sulfonic acid [83-88]. Fig. 10 presents the reaction of amino acids

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Table 4. Comparison of assay methods for cathepsin D [65]. The values given are approximate, and are expressed in terms of the unit of assay I, which cerresponds to about 1,2 ?g human cathepsin D. The estimate of enzyme required per assay assumes that and unincubated blank is required for each chemical method, but is unnecesary in the radiochemical method.

Method	Sample required, miliunit		
	per probe	per assay	concentration/ml
I. E_{280} nm	60	120	120
II. Folin and Ciocalteau + Cu^{2+}	30	60	30
III. Radiochemical	2	2	16

with fluorescamine. The radioelements used for hemoglobin labeling include ^{3}H, ^{14}C, ^{125}I and ^{131}I [90-92]. Addition of a pigment or a fluorizing compound causes substantial changes in globin structure and is frequently associated with reduced susceptibility to cathepsin D action. Addition of a radioelement does not reduce globin susceptibility to cathepsin D (Fig. 11). Moreover, the use of the radioelement-labeled globin requires considerably smaller amount of cathepsin and ensures linear increment of reaction products even at its high activity [5]. Whereas the application of A_{280} nm measurement requires 144 ng and Folina-Ciocalteau's reagents – 72 ng, the radiometric method needs only 4.8 ng of cathepsin D (Table 4).

β-endorphin has been lately recommended as a substrate for cathepsin D activation measurement [93]. This polypeptyde is built up of 31 amino acid residues (Fig. 12). Products of β-endorphin digestion are separated and assessed quantitatively by HPLC technique. Fig. 13 illustrates the differences between cathepsin D and cathepsin E actions on β-endorphin and substance P.

Myoglobin [94], casein [95-97] and albumin [98,99], both labeled and non-labeled with chromofore, fluorophor and radioelement are less frequently used to determine cathepsin D.

The effect of protein digestion by cathepsin D is also assessed by measuring its loss, either without separation or after separation of degradation products. The spectrophometric method [100], the viscosimetric method [101-103], the nephelometric method [104,105] and the plate method [106] are used to assess protein loss without separation of the non-degraded protein from degradation products. In the spectrophometric method, reduction in the number of peptide bonds is evaluated by measuring the absorbance at 225 nm. The viscosimetric method assesses a decrease in viscosity due to protein molecule breakdown into smaller fragments and enables measurement of the activity of endopeptidases contained in a mixture together with exopeptidases which

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H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-
 -Gln-Thr-Pro-Leu-Val-Thr-Leu↓Phe-Lys-Asn-
 -Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
```

Fig. 12. Amino acid sequence of bovine β-endorphin [93]. Arrow indicates atacks by cathepsin D (-Leu17-Phe18-).

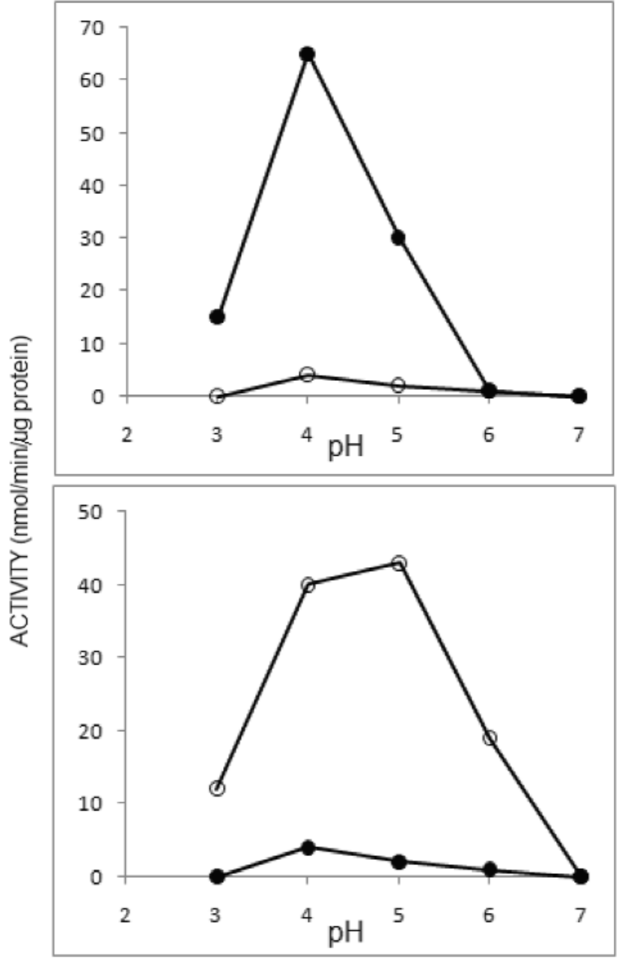

Fig. 13. Dependance on pH of the hydrolysis of β-endorphin (A) and substance P (B) by cathepsins D (•) and E (○) [93].

have no effect on the stickiness of protein molecules. The nephelometric method estimates the intensity of light dispersed after addition of a protein precipitating reagent. The tannin method is the most useful nephelometric method [104]. In the plate methods, such cathepsin D substrates as globin, casein, gelatin or fibrin are placed in a nonsoluble carrier (agar, starch) in Petri plates [4]. A definite volume of the material is placed on plates with a micropipette or filter paper disc saturated with the solution studied. The plates are then incubated at 37°C and after 1-96 hours, depending on the activity of proteinases in the sample, the size of the digested protein field is read directly or after sprin-

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Table 5. Methods for cathepsin D activity measurements using different polypeptides [7,23].

Substrate	Products measurment method
Denaturated hemoglobin	Sectrophotometric (Folin-Ciocalteau reagent + Cu^{2+})
Fluorogen tagged hemoglobin	Fluorimetric
Radioelement tagged hemoglobin	Radiometric
Alkaline protein	Sectrophotometric (Sakaguchi reagent)
Proteoglycans	Wiscosimetric
β-endorfine	High pressure liquid chromatography (HPLC)

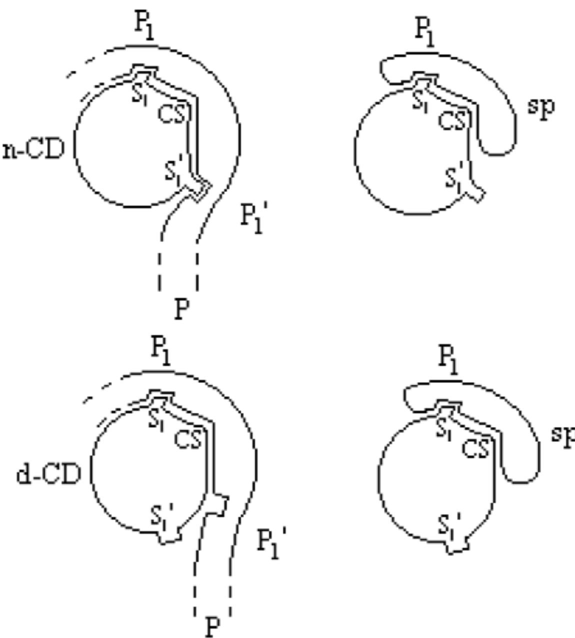

Fig. 14. Binding and hydrolysis of protein (p) and synthetic peptide (sp) by naive cathepsin D (n-CD) and denaturated cathepsin D, bound by α2-M or an antibody (d-CD). S_1, S_1' – substrate binding sites; CS – cathepsin D catalytic site.

kling the plate with a sublimate or sulfosalicylic acid. The plate method is used to detect traces of proteolytic activity, which requires a long incubation time. This method allows a continuous observation of the digestion process. Also other methods can be used to assess the increase in protein degradation products without separation of the non-degraded protein. During protein digestion, the number of amino and carboxyl groups that appear in the non-buffered environment is the measure of enzyme activity [107]. Addition of formol [98] or ethanol reverses dissociation of amino groups, whereas the number of carboxyl groups is determined by titration with titrated NaOH solution, in the presence of phenoloftalein. Acetone causes a decrease in dissociation of carboxyl groups and free amino groups are titrated in this solvent solution using the titrated HCl solution, in the presence of phenol red. Another possibility is precipitation of non-degraded protein by means of copper hydroxide, which at the same time forms soluble complexes with protein digestion products [4]. After filtration, the concentration of color complexes is assessed colorimetrically. Protein loss is also measured after precipitation with TCA acid, washing out of acid, drying and assessing by the weight-based method or after dissolving the sediment in sodium carbonate solution by a chosen method of quantitative protein determination.

The most recommended methods for cathepsin D activity determination using proteins and polypeptides are listed in Table 5.

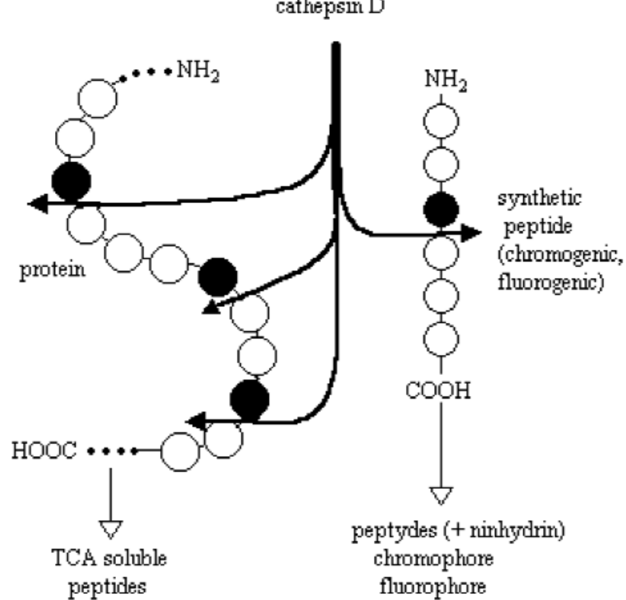

Fig. 15. Products of protein and sythetic peptide degradation by cathepsin D. • – hydrophibic aminoacid rests, ➝ – cleaved peptide bond.

Synthetic peptides

Identification of amino acid residues forming peptide bonds cleaved by cathepsin D in natural peptides helped generate synthetic peptides. Proteins as macromolecular substrates bind to numerous cathepsin D-binding sites (Fig. 14), whereas peptides bind to only one cathepsin D-binding site. Due to this, partly denaturated cathepsin D bound to α2-macroglobulin or to cathepsin D antibodies hydrolyzes peptides but not proteins (Fig. 15).

Cathepsin D hydrolyzes internal peptide bonds in the peptides with at least five amino acid residues in the molecule [108]. These peptides are built up of L-amino acids and contain hydrophobic amino acid

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Detector Group	Spectral Characteristics
para-nitroaniline (pNA) (colorimetric)	Yellow color when cleaved from peptide (405 nm). May also be coupled with p-dimethylamino-cinnamaldehyde to yield a colored schiffbase compound (570 nm).
2-naphthylamine (BNA) (fluorometric or colorimetric)	Fluorometric when cleaved from peptide (excitation 340 nm/emission 425 nm) or Azo coupling reaction with fast blue B as red product (525 nm).
4-methoxy-2-naphthylamine (4 MNA) (fluorometric or colorimetric)	Fluorometric when cleaved from peptide (excitation 340 nm/emission 425 nm) or Azo coupling fast blue B as red product (525 nm).
5-aminoisophthalic acid (AIE) dimethyl ester (fluorometric or colorimetric)	Fluorometric when cleaved from peptide (excitation 335 nm/emission 430nm) or coupling via Bratton Marshall Reaction to yield colored product.
7-amino-4-methylcoumarin (MCA) (fluorometric)	Fluorometric when cleaved from peptide (excitation at 380 nm/emission 460 nm).
7-amino-4-trifluoromethylcoumarin (AFC) (colorimetric or fluorometric)	Yellow color when cleaved from peptide (380 nm) or fluorometric (excitation 400 nm/emission 505 nm).

General Structure of Peptide B-(AA)$_n$-D
Where B (Blocking Group), (AA)$_n$ (Peptide), n (1 to 4 amino acids)
D (Detector Group)

Fig. 16. Function groups used in spectrophotometry and fluorymetry [109].

residues at the site susceptible to cathepsin D action. With peptide chain elongation, the number of grafted peptide bonds increases. The N-terminal amino group and C-terminal carboxyl group of these peptides can be either free or blocked, and may contain prolil residue or D-amino acid residue. In non-fractioned material, the terminal blockage protects these substrates against the action of amino peptidases and carboxyl peptidases.

Spectrophometric or fluorimetric methods are used to determine the products of peptide hydrolysis. Functional groups conditioning the peptide usefulness in spectrophometric and fluorimetric measurements are presented in Fig. 16. Direct and indirect spectrophometry can be distinguished.

In direct spectrophotometry, chromogenic peptide substrates, listed in Table 6, are used. The chromogenic substrates containing the amino acid residue corresponding to cathepsin D specificity, with an attached chromogenic group, allow direct measurement of the amount of the generated product. In the chromogenic substrates, the nitrophenyloalanylic residue is most frequently at the position P1 or P1'. For instance, the course of Phe-Ala-Ala-Phe(NO_2)-Phe-Val-Leu-OM4P hydrolysis leads to generation of Phe-Ala-Ala-Phe(NO_2), which is determined by

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Table 6. Chromogenic peptide substrates for cathepsin D used in direct spectrophotometry.

Peptide	Literature
H-Phe-Gly-His-Phe/NO_2/-Phe-Ala-OMe	Voynick (1971)
H-Phe-Ala-His-Phe/NO_2/-Phe-Val-Leu-OMe	Ferguson (1973)
H-Phe-Gly-His-Phe/NO_2/-Phe-Ala-Phe-OMe	Ferguson (1973)
H-Phe-Gly-His-Phe/NO_2/-Phe-Val-Leu-OMe	Ferguson (1975)
H-Phe-Gly-His-Phe/NO_2/-Phe-Ala-Phe-OMe	Fruton (1976)
H-Leu-Gly-Arg-Phe-Phe-Gly-Gly-OH	Marks (1980)
H-Phe-Ala-Ala-Phe/NO_2/-Phe-Val-Leu-OM4P	Agarwal (1983)
H-Phe-Gly-Gly-Phe/NO_2/-Phe-Val-Leu-OMe	Agarwal (1983)
Pro-Thr-Glu-Phe-Phe/NO_2/-Arg-Leu-OH	Kay (1983)
Glp-His-Phe/NO_2/-Phe-Ala-Leu-NH_2	Pohl (1983)
H-Gly-Gly-His-Phe/NO_2/-Phr-Ala-Leu-NH_2	Pohl (1983)
H-Pro-Thr-Glu-Phe-Phe/NO_2/-Arg-Leu-OH	Dunn (1984)
H-Lys-Pro-Ala-Glu-Phe-Phe/NO_2/-Arg-Leu-OH	Dunn (1985)
H-Pro-Thr-Glu-Phe-Phe/NO_2/-Arg-Leu-OH	Dunn (1986)
Pro-Pro-Thr-Ile-Phe-Phe/NO_2/-Arg-Leu-OH	Jupp (1990)
H-Lys-Pro-Ile-Glu-Phe-Phe/NO_2/-Arg-Leu-OH	Scarborough (1993)
Pro-Thr-Glu-Phe-Phe/NO_2/-Arg-Leu-NH_2	Bolger (1996)
Dansyl-Ala-Gly-Phe/NO_2/-Phe-Gly-OB	Katwa (1996)

absorbance measurement at 310 nm and the results are read from a calibration graph prepared using standard Phe(NO_2) solutions.

The peptides used to determine cathepsin D activity with indirect spectrophometry are listed in Table 7. The reaction products are stained with ninhydrin or by diazotization method. In peptides with blocked N-terminal amino group, the number of amino groups generated via peptide bond hydrolysis is estimated by the ninhydrin method. The course of the reaction is illustrated in Fig. 17. In peptides with the p-aminobenzoic acid residue attached to the C-terminal amino acid, *e.g.* H-D-Phe-Ser-Phe-Phe-Ala-Ala-p-aminobenzoate, cathepsin D cleaves the Phe-Phe bond. The originating Phe-Ala-Ala-p-aminobenzoate is affected by aminopeptidase M (Fig. 18). The diazotization reaction is performed with released p-aminobenzoate (Fig. 19).

The fluorogenic residue is found in fluorogenic substrates at the position P1 or P1' (Table 8). The released fluorophor exhibits optic properties that differ in the emission wavelength compared to the initial compound and can be used for quantitative assessment. As shown in Fig. 20, the fluorescence intensity of Lys-Pro-Leu-Leu-Tyr-Phe(NO_2)-Leu-Leu is very low. The maximum intensity of fluorescence of Phe(NO_2)-Leu-Leu is observed at 303 nm (excitation at 260 nm). In a 12-peptide AMCA-Glu-Glu-Lys-Pro-Ile-Ser-Phe-Phe-Arg-Leu-Gly-Lys(biotinyl)-NH_2, cathepsin D cleaves the Phe-Phe bond. The N-fragment containing AMCA-fluorophor and the C-terminal fragment containing Lys-(biotinyl)-NH_2-fluorofor are formed.

Incorporation of the D-amino acid residue in the peptide structure reduces its susceptibility to the action of cathepsin D. In the hexapeptide Gly-Phe-Leu-Gly-

Table 7. Peptide substrates for cathepsin D in indirect spectrophotometry.

Peptide	Reagent	Literature
N-Ac-Gly-Phe-Leu-Gly-Phe-OH	ninhydrin	Keilova (1971)
Z-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-βNA	diazonium	Reinharz (1971)
Bz-Arg-Gly-Phe-Phe-Leu-4-metoxy-βNA	diazonium	Woessner (19710
Bz-Arg-Pro-Phe-Phe-Leu-4-metoxy-βNA	diazonium	Woessner (1971)
H-D-Phe-Ser-Phe-Phe-ala-Ala-p-Abz	diazonium	Orlowski (1984)
H-Asp-Arg-Val-Tyr-Ile-His-pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OH	ninhydrin after HPLC	Poe (1984)
H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-OH	ninhydrin after HPLC	Cumin (1987)
H-Asp-Val-Arg-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OH	ninhydrin after HPLC	Stammers (1987)
H-D-Phe-Ser-Phe-Phe-Ala-Ala-Abz-OH	diazonium	Adenis (1995)
H-D-Phe-Ser-/O-CH_2-C_6H_5/-Phe-Phe-Ala-Ala-PAB	diazonium	Couissi (1987)

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Fig. 17. The course of the ninhydrin reaction with aminoacids is as follows: 1. ninhydrin (2,2-dihydroxy-1,3-indandione) reacted with amino acid; 2. the intermediate formed as the first reaction product; 3. intermediate gives rise to dipolar ion by decarboxylation and dehydration; 4. the dipolar ion hydrolyzes, producing the amine; 5. the amine condenses with a second molecule of ninhydrin to give Ruhemann's Purple.

Fig. 18. Degradation catalyzed by cathepsin D and aminopeptidaze M [87].

Fig. 19. Diazotization of β-naphtylamine. 1 – β-naphthylamine; 2 – sodium nitrate; 4 – ammonium sulphamate; 6 – ethylenediamine;

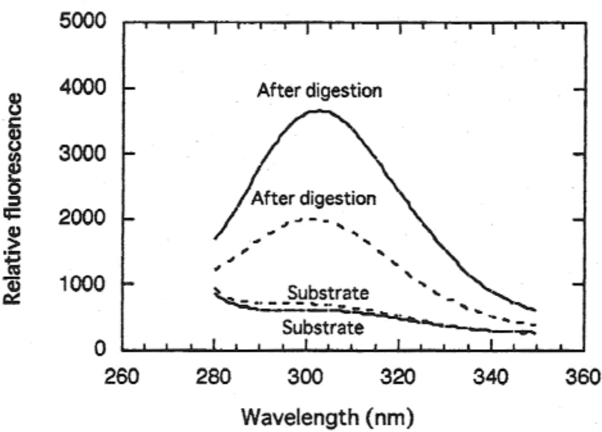

Fig. 20. Fluorescence emission spectra of Lys-Pro-Leu-Leu-Tyr-Phe(NO_2)-Leu-Leu and Lys-Pro-Leu-Leu-Phe-(NO_2)-Tyr-Leu-Leu [146].

Phe-Leu, the Phe-Leu bond is cleaved by cathepsin D [108]. Hydrolysis of hexapeptides having an analogous structure but containing D-amino acids is substantially hindered; besides, they inhibit breakdown of the hexapeptide composed exclusively of L-amino acids. Susceptibility to hydrolysis and degree of inhibition of cathepsin D by these analogues depend on distribution and number of D-amino acids in the molecule (Table 9). The analogues having only one D-amino acid in the molecule, localized at the maximum distance from the site sensitive to the proteinase action, show major susceptibility and poorest inhibitory effect. However, the analogues containing D-amino acid found within the peptide bond that is being cleaved, as well as two amino acids and cyclic hexapeptide are neither hydrolyzed nor exhibit an inhibitory effect [147].

Glycosylation of peptides reduces their susceptibility to the action of cathepsin D. As shown in Table 10, glycosylation of the asparaginian acid residue at the position P4 reduces over fourfold and at the position P4' twofold the susceptibility of the nonapeptide Abz-F-H-L-V-I-H-N-E-EDDnp to the action of cathepsin D. Simultaneous glycosylation at the positions P4 and P4' – makes this peptide resistant to this proteinase.

The synthetic peptides used as cathepsin D substrates are dissolved in dimethylosulfoxide (DMSO), dimethylformamide (DMF) or methanol (Me) [8]. The 1-2% concentration of these solvents does not affect cathepsin D activity. The initial solutions of the substrates have a concentration ranging between 50 and 250 mmol/l. Prior to use, they are dissolved to obtain a concentration of 5 – 25 mmol/l, using a buffer.

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Table 8. Fluorogenic peptide substrates for cathepsin D.

Peptide	Measurement method	Literature
Bz-Arg-Gly-Phe-Phe-Pro-4βNA	fluorimetric	Smith (1975)
Bz-Arg-Gly-Phe-Phe-Leu-4βNA	fluorimetric	Smith (1975)
Abz-Ile-Glu-Phe-Phe/NO_2/-arg-Leu-NH_2	fluorimetric	Bogitsh (1986)
Ac-Glu-Glu/Edans/-Lys-pro-Ile-Cys-Phe-Phe-Arg-Leu-Gly-Lys/DABCYL/-Glu-NH_2	fluorimetric	Baldwin (1993)
Abz-Ile-Glu-Phe-Phe/NO_2/-Arg-Leu-NH_2	fluorimetric	Beyer (1996)
Abz-Ala-Ala-Phe-Phe-Ala-Ala-Ded	fluorimetric	Filipowa (1996)
Abz-Ala-Ala-Phe-Phe-Ala-Ala-pNA	fluorimetric	Filipowa (1996)
Ac-Glu-Asp/EDANS/-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Gly-Lys/DABCYL/-Glu-NH_2	fluorimetric. after HPLC	Gulnik (1997)
NO_2-Tyr-Gly-Se-Thr-Phe-Phe-Lys-Abz	fluorimetric	Peterson (1998)
NO_2-Tyr-Gly-Thr-Gln-Phe-Phe-Lys-Abz	fluorimetric	Petersen (1998)
Abz-Ile-Glu-Phe/NO_2/-Phe-arg-Leu-NH_2	fluorimetric	Verity (1999)
MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys/Dnp/-D-arg-NH_2	fluorimetric	Yasuda (1999)
MOCAc-Gly-His-Pro-Ile-Ile-Phe-Phe-Arg-Leu-Lys/Dnp/-D-arg-NH_2	fluorimetric	Yasuda (1999)
H-Lys-Pro-Leu-Leu-Tyr-Phe(NO_2)-Leu-Leu	fluorimetric	Yonezawa (1999)
H-Lys-Pro-Leu-Leu-Phe(NO_2)-Tyr-Leu-Leu	fluorimetric	Yonezawa (1999)
Abz-Ala-Ile-Ala-Phe-Phe-Ser-Arg-Glu-EDDnp	fluorimetric	Pimenta (2001)
Abz-Ala-Ile-Glu-Phe-Phe-Ser-Arg-Gln-EDDnp	fluorimetric	Pimenta (2001)
Abz-Ala-Ile-Lys-Phe-Phe-ser-Ala-Gln-Thr-Asn-Arg-Gln-EDDnp	fluorimetric	Pimenta (2001)
MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys/Dnp/-Asp-Arg-NH_2	fluorimetric	Komai (2004)
MOCAc-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-arg-Lys-/Dnp/-Arg-Arg-NH_2	fluorimetric	Komai (2004)
AMCA-Glu-Glu-Lys-Pro-Ile-Ser-Phe-Phe-Arg-Leu-Gly-Lys/biotynyl/-NH_2	fluorimetric. after HPLC	Baechle 2005)

Table 9. Cleavage of hexapeptide Gly-Phe-Leu-Gly-Phe-Leu and its diastereomers and inhibition of release of the dipeptyde Gly-Phe from the substrate Gly-Phe-Leu-Gly-Phe-Leu in the presence of its D-isomers by cathepsin D [108].

Hexapeptide*	Cleavage, %	Inhibiton, %
Gly---Phe-Leu-Gly---Phe---Leu	100.0	0.0
Gly---Phe-Leu-Gly---Phe-D-Leu	6.0	89.2
Gly---Phe-Leu-Gly-D-Phe---Leu	3.0	73.4
Gly-D-Phe-Leu-Gly---Phe---Leu	0.0	63.1
Gly-D-Phe-Leu-Gly-D-Phe---Leu	0.0	0.0
Gly---Phe-Leu-Gly---Phe---Leu	0.0	0.0

* – arrow indicates cleaved peptide bond

Table 10. Kinetic constants for hydrolysis of internally quenched fluorescent peptides by human cathepsin D [148]. Arrows indicate the cleavage site, NDH – no detected hydrolysis.

Sunstrates-P1-P1'-	Cathepsin D, kcat/Km
(GlcNAcβ) Abz-N-F-H-L- V-I-H-N-E-EDDnp	113
(GlcNAcβ) Abz -F-H-L- V-I-H-N-E-EDDnp	263
(GlcNAcβ) (GlcNAcβ) Abz-N-F-H-L- V-I-H-N-E-EDDnp	NDH
Abz-F-H-L-V-I-H-N-E-EDDnp	454

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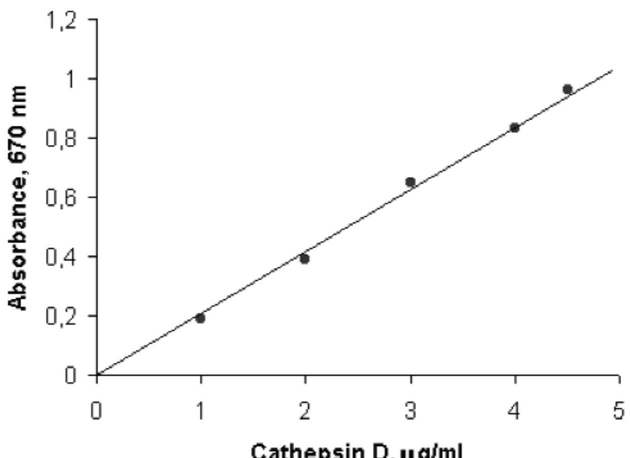

Fig. 21. Hydrolysis of hemoglobin by cathepsin D as function of enzyme concentration.

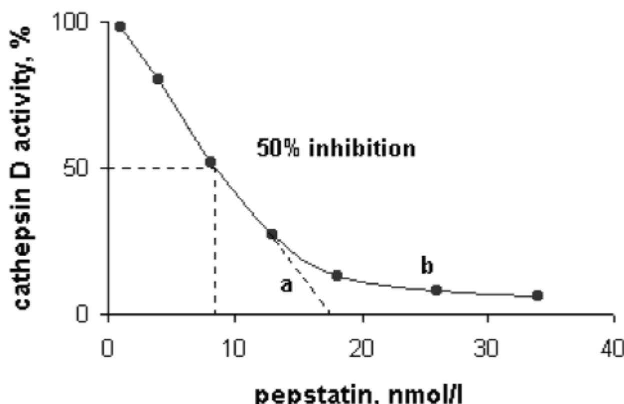

Fig. 22. Inhibition curve obtained by addition of increasing amounts of pepstatin to the cathepsin D; substrate – hemoglobin; titration curve: a – ideal, b – real [149].

Determination of concentration

The concentration and content of active cathepsin D molecules in blood plasma and tissue homogenates are determined based on the results of its activity measurement and the calibration graph presenting the correlation between the activity and concentration (Fig. 21). Titration of a catalytic site using pepstatin [149] or dansyl-pepstatin [150], and placing the results on the graph to help read the concentration value is another way used to determine active cathepsin D molecule concentration (Fig. 22).

Total concentration and content of both inactive and active cathepsin D are determined using specific antibodies by the immunoenzymatic method ELISA [151,152] and by radioimmunoenzymatic methods [153,154] (Table 11).

Cathepsin D possesses a few sequential and conformational antigen determinants, against which antibodies are produced. Antibodies contained in anti-

Table 11. Immunoenzymatic methods for estimation of cathepsin D concentration.

Method	Reagent (company)
Immunenzymatic methid (ELISA)	Enzyme-linked immunosorbent assay Kit (Ciba Corning Diagnostic, Italy; Triton Diagnostics, USA).
Rapid immunoenzymatic method (ELISA)	Cathepsin D Rapid Format ELISA assay Kit (Calbiochem, Suisse)
Immunoradiometric method (IRMA)	Immunoradiometric assay IRMA Cath-D Kit (Cis Bio-International, Gif-Sur-Yvette, France)

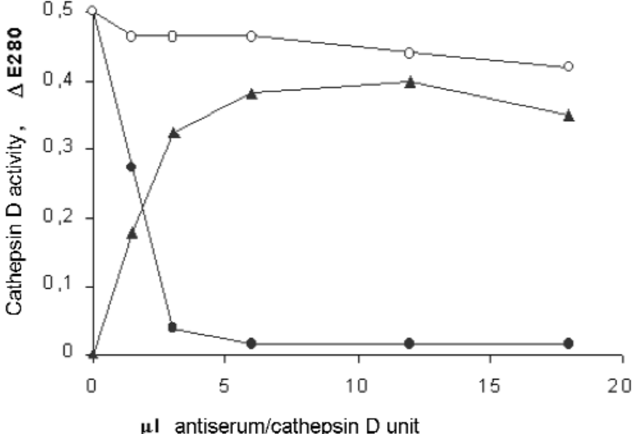

Fig. 23. Effect of antiserum ratio on cathepsin D activity in pH=3.2 [159,160]. ○ – before centrifuging; ● – in supernatant; ▲ – in precipitate.

serum, isolated antibodies and monovalent Fab (fragment antigen binding) fragments of immunoglobulins bind to cathepsin D [155-158]. Formation of bonds between cathepsin D and antibodies is most intense at pH above 3.0-5.0. Antigen determinants and a catalytic site of cathepsin D are located at distant sites of the molecule. This, however, does not hinder access of the substrate to the catalytic site. Only removal of the cathepsin D-antibody complex from the solution by centrifugation leads to the loss of activity in the supernatant (Fig. 23). Fig. 24 illustrates the principle of the immunoenzymatic method, whereas Fig. 25 shows the principle of the radioimmunoenzymatic method of cathepsin D assay.

Determination of tissue and cell location of cathepsin D

Tissues and organs differ in cathepsin D content [2,93]. Table 12 shows the content and activity of this enzyme in the rat organs. Cell composition differs between organs as well as between healthy and pathological tissues. The liver is characterized by high

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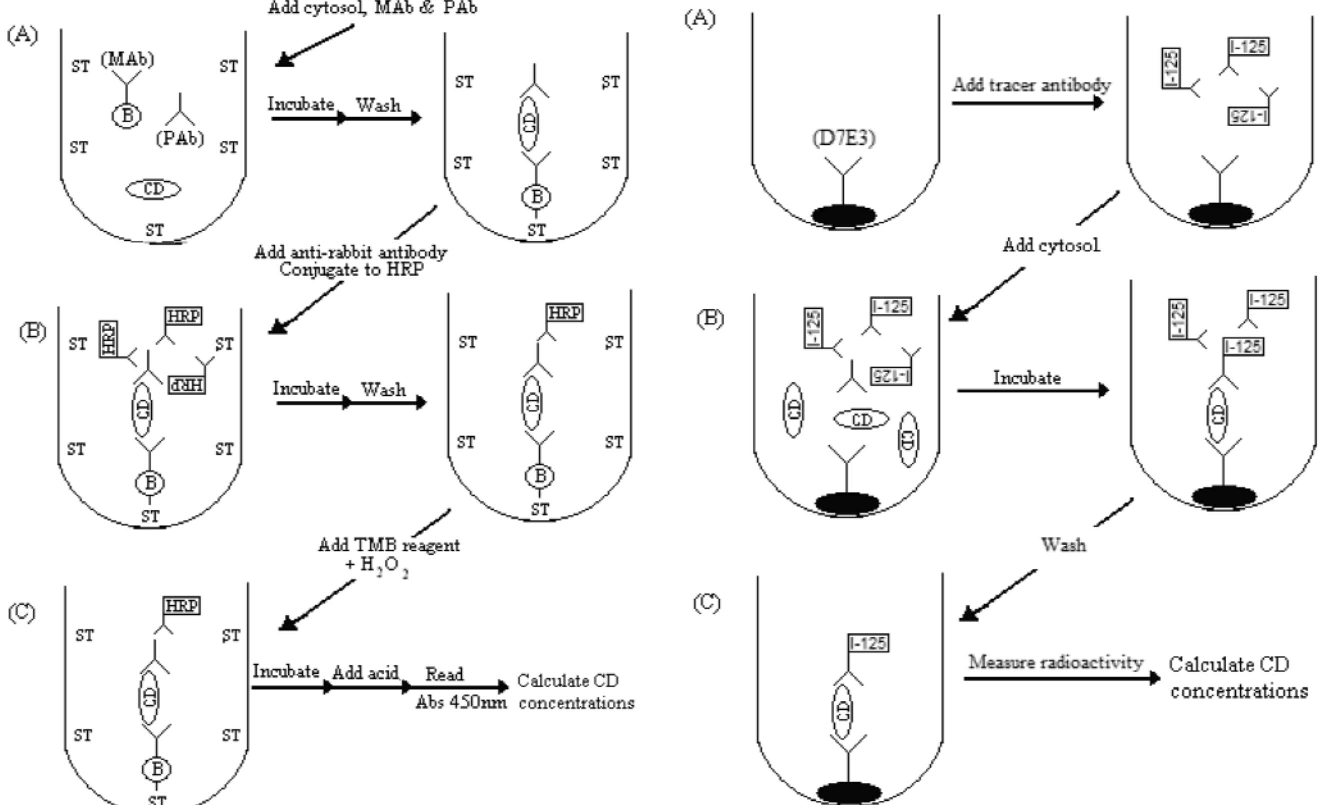

Fig. 24. Schema for performing the immunoassay of cathepsin D [161].

Fig. 25. Schema for performing the immuno-radiometric assay of cathepsin D [161].

diversity of cell composition. The respective types of cells in this organ show varied cathepsin D activity (Table 13). Among the cells that build up the liver, Kupfer cells exhibit the highest cathepsin D activity. Cathepsin D is localized in hepatic lysosomes (Table 14) that account for 1% of total hepatocyte volume, their number ranging from 300 to 400.

In cytomechanical and histochemical techniques, it is postulated that the morphological structures should be maintained unchanged so that they could be recognized microscopically. To do this, the cell and tissue materials need to be properly prepared. Therefore, the classical fixation methods are replaced by the cryostat sectioning technique. This procedure is the method of choice, especially in histoenzymology, for the performance of enzymatic reaction. The final product of the reaction is perceptible under optical or electron microscopes.

Cathepsin D can be localized in cells and tissues using cyto/histochemical and immunocyto/histochemical techniques [163-170]. The former employ methylmercury pepstatin derivative, biotin-labeled pepstatin and biman-labeled pepstatin [171-174], as well as chemical compounds shown in Fig. 26. In the latter techniques, tissue sections are fixed using a set of reagents AMeX (acetone, methyl benzoate, xylene) [175,176]. Polyclonal antibodies (DAKO A/S Glostrup, Denmark) bind to cathepsin D epitopes. Cathepsin D is visualized by means of avidin-biotin peroxidase complex (ABC) kit [177,178]. 3-amino-9-ethylcarbazole (AEC) is used as chromogene, yielding a red color reaction product.

Determination of activity and concentration of cathepsin D inhibitors

Cathepsin D has neither endogenous lysosomal nor cytosolic inhibitors. When lysosomes become damaged and cathepsin D passes to the intracellular fluid and blood plasma, it is neutral pH and α2-macroglobulin (α2-M) that prevent uncontrolled proteolysis [179,180].

α2-M is synthesized in fibroblasts and then passes to the intracellular fluid, lymph and blood. In plasma, α2-M occurs in a concentration of 260.0 mg/100 ml (3.3 μmol/l). Approximately 20% of plasma cathepsin D is bound to α2-M [181,182]. The remaining 80% occurs in a free state as procathepsin D, the inactive precursor not bound to α2-macroglobulin. The cathepsin D binding to pepstatin hinders interaction with α_2-macroglobulin, due to which the amount of cathepsin D bound to this inhibitor decreases to approximately

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Table 12. Levels of cathepsin D in rat and mokey tissues.

Rat		Tissue		Monkey	
Hydrolytic activity βE nmol/min/mg tissue protein	CTSD ng/mg tissue protein			Hydrolytic activity βE nmol/min/mg tissue protein	CTSD ng/mg tissue protein
14.0	220	cerebrum	frontal cortex	8.1	140
-	-	-	hypothalamus	6.3	110
-	-	-	pituary	11.0	190
8.2	130	cerebellum		9.3	160
14.0	190	thymus		6.4	110
17.0	270	lung		5.6	98
4.4	69	heart		2.5	44
9.3	150	liver		3.9	68
12.0	99	stomach		-	-
3.5	49	duodenum		15.0	250
4.2	61	jejunum		40.0	700
4.2	59	ileum		20.0	350
4.3	65	colon		23.0	400
23.0	330	spleen		36.0	630
12.0	190	kidney		4.0	70
69.0	1090	adrenal		45.0	790
5.6	88	pancreas		8.1	140
12.0	150	urinary bladder		30.0	530
2.5	39	muscle		0.62	11
12.0	180	lymph node		43.0	750
7.8	98	bone marrow		5.6	97
0.05	<1	serum		0.01	<1
<0.01	<1	blood cells	erythrocyte	5.3	93
-	-	-	leucocyte	<0.01	<1

Table 13. Activity of cathepsin D in perenchymal, sinusoidal, endothelial and Kupffer cells isolated from the rat liver [162].

Cells suspension	Cathepsin D activity, nmoles tryptophan	
	nmoles/min/ mg protein	nmoles/min/ 10^6 cells
Perenchymal cells	0.52	0.66
Sinusoidal lining cells	8.44	0.51
Endothelial cells	6.12	0.25
Kupffer cells	17.97	2.11

Table 14. Intracellular distribution of cathepsin D in rat liver [36]. Ex – cytoplasmic extract, N – nuclear fraction, M – mitochondrial fraction, L – lysosomal fraction, Mic – microsomal fraction, S – soluble fraction (final supernatant).

Fractions	Absolute values		Percentage values	
	Nitrogen mg/g	Cathepsin D units/g	Nitrogen	Cathepsin D
Ex + N	32.3	1.46	100	100
N	4.3	0.06	13.3	4.0
M	6.2	0.51	16.3	35.0
L	2.4	0.63	7.4	42.9
Mic	7.9	0.11	24.4	7.5
S	12.2	0.25	37.5	17.1

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Fig, 26. Diagram representing the three chemistries for the cytochemical staining of proteases that liberate 4-metoxy-β-naphthylamine.

8%. The concentration of α_2-M is determined with antibodies by the nephelometric method, laser nephelometry and radial immunodiffusion. The activity of α2-macroglobulin is assessed by the enzymatic method by measuring the reduction in proteolytic activity in a standard testing system [183]. Cell and tissue α_2-M is also localized. Binding to α2-macroglobulin inhibits the action of cathepsin D on macromolecular substrates and only slightly on micromolecular substrates. The inhibitory activity of α2-macroglobulin towards cathepsin D occurs at pH 5.5-6.0, but not at a lower pH because of structural instability [185]. α2-macroglobulin is inactivated with methylamine [186,187]. This proteinase inhibitor prevents uncontrolled proteolysis [180] via binding, inhibition and removal of proteinases from plasma and intracellular fluid. The α2-M-proteinase complexes are captured by specific macrophage receptors [188], undergo endocytosis and are transported into lysosomes. Then, the complexes undergo digestion and the receptors return to the cell surface.

The α2-M differs from typical proteinase inhibitors in binding mode and low specificity. The catalytic site of cathepsin D bound to α2-M is free. The micromolecular substrates and inhibitors, but not the macromolecular ones, have access to this site. Cathepsin D and α2-M show a two-stage interaction: in the first stage, cathepsin D cleaves the specific peptide bonds situated within the polypeptide chain of the inhibitor, thus causing conformatory changes within the inhibitor; in the second stage – the enzyme is surrounded and access of macromolecular substrates is blocked. Exopeptidases cannot cleave the peptide bonds situated within the α2-M polypeptide chain and thus their activity is not blocked by this inhibitor.

Exogenous cathepsin D inhibitors include such synthetic compounds as 1,2-epoxy-3-(p-nitrophenoxy)-propan, diazoacetyl-dl-norleucin methyl ester, pepstatin and its derivative [116,189] and peptide inhibitors found in spare organs of many plant species [116,190,191]. The activity of exogenous cathepsin D inhibitors is determined in a test in which: 1/ cathepsin releases the reaction product in the amount corresponding to the absorbance of 0.50; 2/ the inhibitor blocks 50% of enzyme activity. The inhibitor activity is calculated from the formula: inhibitor, U/ml/min = (d x r) : t, in which: d means difference in the quantity of the reaction products in a test without and with inhibitor, r – inhibitor dilution, t – incubation time. The inhibitor sample is diluted when activity suppression exceeds 50%. The septopeptide Pro-Thr-Glu-Phe-Phe(NO_2)-Arg-Leu [116] or globin [191] is used to determine the inhibitor activity.

The enzyme- inhibitor molar ratio is determined from the plot presenting the correlation between cathepsin D activity and inhibitor concentration [192,193]. The inhibitor concentration causing a 50% reduction in the enzyme activity, referred to as the inhibitory dose 50 (ID_{50}), is taken into consideration in the assessment and expressed in μmol/l [189]. The numerical value of ID_{50} is used to compare the strength of the inhibitory effect of various inhibitors. The therapeutic application of the inhibitor is preceded by determination of its toxicity expressed as the lethal dose 50 (LD_{50}), as established for experimental animals [194].

Determination of the effect of preincubation time with inhibitor on the measurement shows its immediate or progressive action. The measurement of inhibitor activity can also be affected by the sequence in which reagents are added: inhibitor – enzyme – sub-

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strate or inhibitor – substrate – enzyme [196,197]. Determination of the activity of cathepsin D inhibitors in non-fractionated extracts from cells of microorganisms and plant tissues can be burdened with error, as these samples may also contain, apart from these inhibitors, proteinases that function in an acidic pH [180]. Prior to homogenization, the inhibitor and proteinase are found in different cell compartments and come into contact only after homogenization. These proteinases can be both sensitive and insensitive to the inhibitors and in both cases reduce the measurement, either suppressing the inhibitor activity or leading to its proteolytic inactivation, respectively. These proteinases can also degrade and inactivate cathepsin D. In the case of stable micromolecular inhibitors (plant extract, homogenate, plasma, urine), proteases are inactivated thermally or with trichloroacetate acid or perchloric acid. The precipitated proteins are eliminated via centrifugation. Trichloroacetate acid is removed from the supernantant by ether extraction, whereas perchloric acid is precipitated using potassium hydroxide. The inhibitor concentration is determined based on the antiproteolytic activity. The calibration graph is drawn to show the correlation between cathepsin D activity and inhibitor concentration. Determination of cathepsin D activity after addition of a deproteinized sample allows reading the concentration of the inhibitor.

In studies on cathepsin D inhibitors, especially in plant samples, the presence of nonspecific cathepsin D inactivators, including polyphenol compounds, should be excluded [198].

Diagnostic significance of cathepsin D

In physiological conditions, only small amounts of cathepsin D pass to the intercellular environment, to blood and body fluids via exocytosis of procathepsin and cathepsin D, excretion of residue bodies and disintegration of physiologically exhausted cells [199-201]. The pathological conditions, occurring with hypoxia, acidemia and especially necrosis, are characterized by damage to lysosomal and cellular membranes, and release of increased amounts of cathepsin D [202]. Enhanced synthesis of cathepsin D and insufficiency of the intercellular apparatus transporting procathepsin may contribute to the increased escape of cathepsin D out of cell [203-205]. The activity of cathepsin D in plasma depends also on its uptake by monocytes/macrophages in the liver and spleen [202-206]. An impaired uptake or saturation of these cells with phagocytized material may cause a rise in plasma cathepsin D activity. The half-life of cathepsin D in blood amounts to 60-90 minutes [207-208].

For diagnostic purposes, the activity and level of cathepsin D are assessed in homogenate (total activity), in the cytoplasmic fraction, in the lysosomal fraction, in blood plasma, in secretions (saliva, gastric juice), excretions (urine, feces), and body fluids (peritoneal fluid, pleural fluid, cerebrospinal fluid).

The activity/level of cathepsin D and its inhibitors in plasma and body fluids are calculated per cell count in solid tissues per 1g of wet or dry tissue or 1 μg of DNA [209-211]. Determination of cathepsin D activity in homogenate per ml of protein for diagnostic purposes or in toxicological investigations is not justified and may lead to interpretation errors. With the same activity of cathepsin D in homogenate – the activity is high when the protein content is low, and the activity is low when the protein content is high. Measurement of cathepsin D activity per ml of protein is useful only for preparatory tasks and when purification degree of this proteinase is calculated.

Also other endopetidases involved in protein digestion at an acidic pH can be found in plasma and tissue homogenates, namely cathepsin B and cathepsin L (pH 5.5), cathepsin E (pH 2.5) and pepsin (pH 2.0) [212-214]. The pH ranges in which these enzymes function are found to overlap. The specificity of cathepsin D determination increases following addition of the cathepsin B and L inhibitor, *i.e.* L-trans-epoxysuccinyl-Leu-4-guanidinobutylamide (E-64), and the cathepsin E and pepsin inhibitor isolated from Ascaris lumbricoides (AlI) to the incubation mixture. The activity of cathepsin D is lower in plasma than in serum [247]. Hemolysis does not affect the measurement. When an immunological method is used, other cathepsins have no effect on the determination of cathespin D concentration/content. The lower limit of cathepsin D measurability is 0.012 nmol/l. Plasma cathepsin D level should range from 9.9 nmol/l (healthy women) to 10.6 nmol/l (healthy men) [218,219].

The activity and concentration/level of cathepsin D are frequently found to overlap. However, sometimes the opposite situation may occur. The activity may be higher than the concentration when conformation masking or sequential epitopes condition the reactions with antibody, or lower when the molecule undergoes inactivation (denaturation) without impairment of its epitope.

The histochemically and immunohistochemically determined distribution of cathepsin D and its inhibitors (mainly α2-M) in cells and tissues are presented on color microphotographs. The reaction intensity is assessed semiquantitatively using the following scale: lack of reaction (-), weakly positive reaction (±), positive reaction (+), strongly positive reaction (++).

Conclusion

Determination of the activity/concentration and cell/tissue distribution of cathepsin D and its inhibitors plays a major role in biochemistry, pathobiochemistry and diagnostics. Development of analytical methods

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involves a search for specific and sensitive substrates (beta-endorphin, synthetic fluorogenic peptides) and new analytical techniques: HPLC [145,220], capillary electrophoresis [221-223], fluorimetry in the near infrared region [224], flow cytofluorimetry [225-229], western blot [230-232], immunohistochemical techniques [168,169], fluorescence microscopy [233,234] and electron microscopy [235,236].

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