

Cathepsin D inhibitors

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Abstract: Inhibitors of cathepsin D belong to chemical compounds that estrify carboxyl groups of the Asp33 and Asp231 residues of its catalytic site, penta-peptides containing statin, *i.e.* the amino acid similar in structure to the tetraedric indirect product, and polypeptides found in the spare organs of many plants and forming permanent noncovalent complexes with cathepsin. Cathepsin D activity is also inhibited by alpha2-macroglobulin and antibodies directed against this enzyme. Methods used to determine the activity and concentration of these inhibitors and their analytical, preparative and therapeutic applications are discussed.

Key words: Cathepsin D - Inhibitors - Aspartyl endopeptidase

Cathepsin D (EC 3.4.23.5) is an aspartyl endopeptidase, like cathepsin E, pepsin, gastrixin and rennin. Aspartyl endopeptidases are synthesized by malaria sporozoites, sheep liver fluke *Fasciola hepatica*, fungi *Candida albicans* and human immunodeficiency virus HIV [1-5]. Cathepsin D is synthesized in the rough endoplasmic reticulum (RER) in a form of preprocathepsin D, built up of 412 amino acid residues [6,7]. The RER is the site of glycosylation and formation of disulphide bridges in preprocathepsin D. Splitting off of the N-terminal 20 amino acid prepeptide by signalase causes release of procathepsin D and its passage to the Golgi apparatus and primary lysosomes. In an acid environment of the primary lysosomes, intramolecular autocatalytic splitting off of the 44 amino acid propeptide and the formation of active enzyme occur [8-12]. The actions of cysteine proteases, aminopeptidases, carboxypeptidases and the autocatalytic process lead to the formation of a mature two-chain cathepsin D molecule, 12 + 34 kDa, [13,14]. These protease inhibitors inhibit maturation of cathepsin D [15], which can also be found on the cell surface due to fusion of lysosomes with the plasma membrane [16,17]. Cathepsin D may pass to cytosole, to the intracellular environment and to the blood.

The catalytic site of cathepsin D consists of two aspartic acid residues: Asp33 and Asp231 [18], which together with water molecule cause hydrolysis of peptide binding. The ionized carboxyl group of the Asp33 residue activates the water molecule and facilitates proton detachment. The protonated carboxyl group of the Asp231 residue, in an acid environment, polarizes the carboxyl group of the substrate peptide binding, facilitates formation of indirect tetraedric product and increases its vulnerability to cleavage. The cleavage refers to the peptide binding situated within the polypeptide chain formed by carboxyl groups of hydrophobic amino acids [19].

Cathepsin D is involved in the process of degradation of exploited and denaturated cell proteins and those reaching the cell via endocytosis [20,21]. It also affects proteins indirectly through activation of procathepsin B [22,23], procathepsin L [24] prorenin [25] and inactivation of cystatins [26,27], stephins [26], and inhibitors of seryl protease type 1-2 [28], urokinase type 1-3 [28, 29], α 1-antithrypsin [30], α 1-antichymothrypsin [31] and calicrein [31]. Cathepsin D is also engaged upon selective proteolysis, which leads to the activation of prohormons and biologically active peptides and to the inactivation of their active forms [16,27,32-37]. Moreover, it takes part in the formation, development and regression of such pathological changes as inflammatory states, atherosclerosis, intravascular thrombi, apoptosis, mutagenesis, neoplastic proliferation or Alzheimer disease [38-53].

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Cathepsin D activity is regulated on a few levels: through stimulation and inhibition of its biosynthesis, at the stage of post-translatory modifications in the Golgi apparatus, proenzyme activation and interlysosomal pH regulation [54-56]. This ensures that cathepsin D can operate at the proper time, site and intensity, and prevents uncontrolled protein degradation. The 20-kDa cytoplasmic protein, glycine ethyl ester, tripolyphosphates, phospholipids and ATP increase the activity of cathepsin D [57-59].

Cathepsin D degrades proteins at pH=3.5-5.5 [60]. Binding of this protease to a constant carrier shifts digestion towards neutral pH [61]. Vulnerability to degradation by cathepsin D depends also on the protein molecular structure. Haemoglobin and other denaturated proteins are more prone to the action of this protease than native proteins. However, complexification of haemoglobin with haptoglobin causes a reduction in the optimum pH from 3.5 to 2.8 and at the same time a 50% decrease in degradation rate [62].

Definition and division of cathepsin D inhibitors

The inhibitor is a compound showing chemical affinity toward an enzyme or bearing structural similarity to a substrate which binds to the catalytic site of an enzyme and forms an inactive enzyme-inhibitor complex. Chemical compounds reducing the enzyme activity via another mechanism are referred to as inactivators. Inactivators do not exhibit specificity for a particular enzyme or group of enzymes and include such protein-denaturing agents as heavy metal salts, alkaloid reagents, tannins, detergents, acids and bases, and elevated temperature. The inhibitors can be divided into competitive, noncompetitive and mixed, immediate and progressive, monovalent and polyvalent, synthetic and natural, endogenous and exogenous. The Lineweaver-Burk equations and plots are used to determine the competitive, noncompetitive and mixed types of inhibitors [63-66]. Based on the experimental data obtained for various inhibitor concentrations, the Michaelis constant and the maximum velocity are determined. The values obtained are placed in a plot showing the relationship of the reciprocal of the Michaelis constant and the reciprocal of the maximum velocity with the inhibitor concentration. Depending on the type of inhibitor, the curves may show either increased inclination or a shift of the point of intersection with the axis of ordinates. The competitive inhibitor shows structural similarity to a substrate with which it competes for an enzyme's catalytic site. It reduces substrate affinity for an enzyme and increases the Michaelis constant ($1/K_m$). However, the maximum velocity, under substrate excess, remains stable ($1/V_{max}$). This causes increased inclination of the

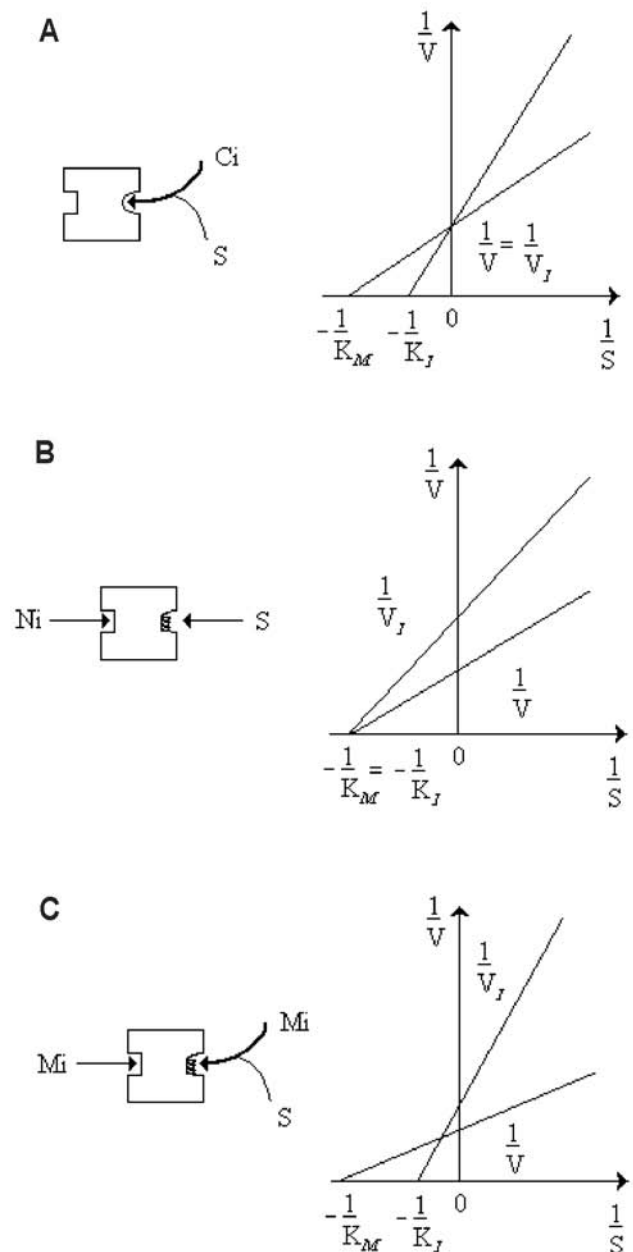


Fig. 1. Determination of the inhibition constant by the double-reciprocal plot method; **A** - competitive inhibition, **B** - noncompetitive inhibition, **C** - inhibition of the mixed type; Ci - competitive inhibitor, Ni - noncompetitive inhibitor, Mi - mixed type inhibitor [63].

Lineaweaver-Burk plot, at the same point of intersection with the axis of ordinates as when the inhibitor is absent (Fig. 1A). The noncompetitive inhibitor has the substrate structure and binds to the enzyme outside the catalytic site. Such an inhibitor does not alter the Michaelis constant ($1/K_m$), but decreases the maximum reaction velocity ($1/V_{max}$), which leads to increased inclination of the curve and to a shift of the point of intersection with the axis of ordinates (Fig. 1B). The mixed inhibitor, which is partly competitive

Table 1. Properties of different types of inhibitors: competitive, noncompetitive and mixed type.

Property	Inhibitor type		
	competitive	noncompetitive	mixed type
Structural similarity to substrate	similar	not similar	partly similar
Binding site	catalytic site	outside catalytic site	catalytic site and outside catalytic site
Reversibility through substrate surplus	reversible	not reversible	partly reversible
V_{max}	none	decrease	decrease
K_m	increase	none	increase

Table 2. Inhibitor susceptibility of proteases with different structure of catalytic site. x - L-3-carboxy-2,3-trans-epoxy-propionyl-leucylamido(guanidino)butane; xx - 3,4-dichloroisocumarine.

Proteases	Inhibitor
Aspartyl	pepstatin
Cysteinyll	E-64 ^x
Seryl	3,4-DCI ^{xx}
Metalloproteases	1,10-phenantroline

release of the reaction product. Mixed type inhibition can be also observed when cathepsin D exerts a simultaneous effect on different substrates, including a mixture of protein partial degradation products having varied affinity for this protease [67]. The inhibitor acts competitively toward some substrates and noncompetitively toward others. Characteristic features of various types of inhibitors have been presented in Table 1.

Pepstatin acts as an inhibitor of cathepsin D and other aspartyl proteases (Table 2). It does not inhibit

Table 3. The inhibitory effect of pepstatin A, antipain, leupeptine and chymostatin on cathepsins A, B and D [68].

Cathepsin	Substrate	IC ₅₀ (µg/ml)			
		Pepstatin A	Antipain	Leupaptine	Chymostatin
A	Cbz-L-Glu-L-Tyr	125.00	1.20	1680.00	62.5
B	Bz-l-Arg-NH ₂	125.00	0.60	0.40	2.60
D	Hemoglobina	0.01	125.00	109.00	0.011

Table 4. Vulnerability of human aspartyl proteases to inhibitors. * + inhibitory effect, - lack of inhibition.

Inhibitor*	Cathepsin D	Cathepsin E	Pepsin	Renin
Pepstatin A	+	+	+	+
<i>Ascaris sp.</i> derived inhibitor	-	+	+	-
Antibodies against cathepsin D	+	-	-	-

and partly noncompetitive, binds to free enzyme and to the enzyme-substrate complex. In the Lineweaver-Burk plot, both the Michaelis constant (1/K_m) and the maximum velocity (1/V_{max}) increase (Fig. 1C). Mixed inhibitors hinder the formation of the enzyme-substrate complex and decrease the velocity of formation and

the activities of seryl cathepsins, cysteinyl cathepsins or metalloproteases, and their inhibitors do not block the activity of cathepsin D. A properly chosen set of inhibitors allows differentiation of cathepsin D from cathepsin A and B (Table 3), and from cathepsin E, pepsin and rennin, which are also aspartyl proteases (Table 4). Dithiotreitol, at a concentration of 1 mmol/l, pH=8.0, inactivates this cathepsin [69]. However, endogenous thiols, at physiological concentrations, do not affect cathepsin D activity.

The propeptide split off of procathepsin D during activation [70,71], α₂-macroglobulin [72] and DNA fragments [73] are endogenous cathepsin D inhibitors. Their inhibitory effects are slight and observed only in special conditions. Most cathepsin D inhibitors are synthetic, peptide and polypeptide produced by microorganisms, plants and animals [67,74-77]. Specific anti-cathepsin D antibodies also have an inhibitory effect.

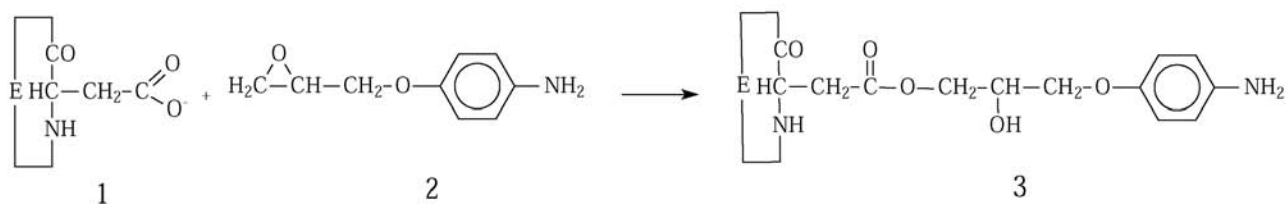


Fig. 2. Reaction of cathepsin D Asp33 ionized residue (1) with 1,2-epoxy-3-(p-nitrophenoxy)propane (2); (3) 2-hydroxy-3-(p-nitrophenoxy)propyl ester [63].

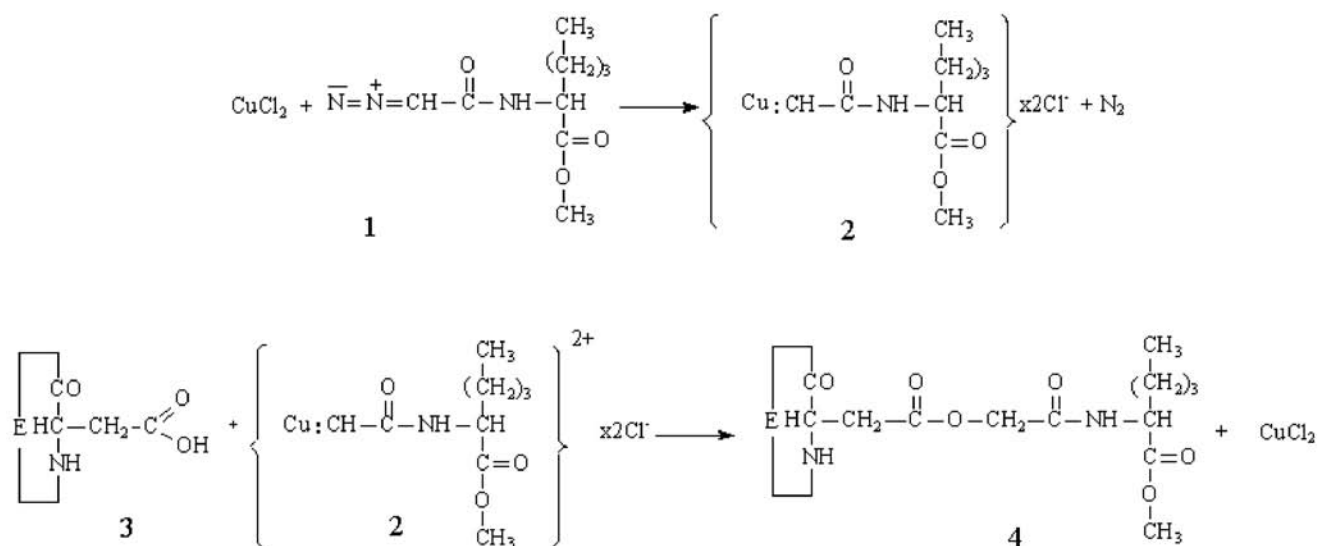


Fig. 3. Reaction of cupric chloride with diazoacetyl norleucine methyl ester (1) with creation of carbene with cupric ions complex chloride (2); nonionised Asp231 residue of cathepsin D (3) with carbene with cupric ions complex chloride (2) with creation of 2-oxo-3-ase-4-metoxycarbonyloxy ester (4) [63].

Synthetic inhibitors

Synthetic inhibitors of cathepsin D are micromolecular organic compounds estrifying the carboxyl group of the Asp33 or Asp231 residue in the catalytic site. The Asp33 residue reacts with 1,2-epoxy-3-(p-nitrophenyloxy)propan (Fig. 2). The compounds reacting with the Asp231 residue include diazoacetyl derivatives of amino acid methyl esters. The carboxyl groups of cathepsin D are estrified by these compounds in the presence of copper ions having a catalytic function [78]. The reaction of cathepsin D with diazoacetyl norleucine methyl ester has been presented in Fig. 3. Other cathepsin D inhibitors are: diazoacetyl-glycine ethyl ester [79], diazoacetyl-phenylalanine methyl ester [80], diazoacetyl-2,4-dinitrophenyl-ethylenediamine [81] and other diazole compounds [81-85]. The reaction of cathepsin D with diazole compounds occurs most rapidly at pH=4.5. Other cathepsin D inhibitors also include many derivatives of 4-(morpholinylsulphonyl)-L-Phe-P₂-(cyclohexyl)Ala[isostere]-P₁'-P₂', listed in Table V. Also ditiophosgen and 2,2-dichloro-1,3-dithiocyclobutanone [87], and methyl blue

and tetranitromethane [88] have been found to inhibit cathepsin D activity. These compounds inactivate pepsin as well. However, 2,4'-dibromoacetophenone and 2-bromo-2-phenylacetophenone inhibit pepsin but not cathepsin D activity [78,89,90].

Synthetic substrate analogues

Cathepsin D activity is inhibited by structural analogues of synthetic substrates. These are oligopeptides containing at least five amino acid residues in the molecule and having L-amino acid replaced by D-amino acid. In the pentapeptide Pro1-Phe2-Phe3-Val4-Leu5, cathepsin D causes the cleavage of the Phe2-Phe3 bond [91]. Replacement of L-Leu5 residue by D-Leu5 makes this pentapeptide resistant to the action of cathepsin D and able to inhibit hydrolysis of matrix pentapeptide. In the Gly1-Phe2-Leu3-Gly4-Phe5-Leu6 hexapeptide, cathepsin D causes the cleavage of the Phe2-Leu3 bond. The hydrolysis of peptides of analogous structure but containing D-amino acids in positions P1, P3 or P4 is considerably hindered; moreover they inhibit decomposition of a hexapeptide built

Table 5. Inhibition constant (K_i) of 4-(morpholinylsulphonyl)-L-Phe-P2-(cyclohexyl)Ala(isostere)-P1-P2 derivatives, in relation to human aspartyl proteases [86]. Abbreviations: ACFHP - 4-amino-5-cyclohexyl-2,2-difluoro-3-hydroxypentanamides; AEM - 2-morpholinylethylamide; MBA - 2-methylbutylamide; ACFOP - 4-amino-5-cyclohexyl-2,2-difluoro-3-oxopentanamides; 3,4-ACDMH - 2-amino-1-cyclohexyl-3,4-dihydroxy-6-methylheptane; ACDPD - 2-(2-amino-1-cyclohexyl-3,5-dihydroxypent-6-yl)-1,3-dithiane; 3,5-ACDMH - 2-amino-1-cyclohexyl-3,5-dihydroxy-6-methylheptane; R-ACDH - 5(R)-2-amino-1-cyclohexyl-3,5-dihydroxyheptane; S-ACDH - 5(S)-2-amino-1-cyclohexyl-3,5-dihydroxyheptane.

P_2	P1-P1'	K_i , nmol/l			
		pepsin	gastricsin	cathepsin D	cathepsin E
allyl	ACFHP-AEM	1240.0	>4000.0	0.8	21.0
allyl	ACFHP-MBA	27.0	13.0	3.2	0.1
allyl	ACFOP-AEM	11.0	0.9	18.0	0.5
allyl	ACFOP-MBA	24.0	9.0	0.4	0.5
allyl	3,4-ACDMH	13.0	8.0	2.5	17.0
allyl	ACDPD	16.0	>4000.0	0.6	2.2
allyl	3,5-ACDMH	57.0	19.0	0.1	2.0
allyl	R-ACDH	77.0	>4000.0	5.4	11.0
allyl	S-ACDH	69.0	>4000.0	6.1	3.3
allylthio (R/S)	S-ACDH	516.0	>4000.0	15.0	2.9
CH ₂ CO ₂ Me	R-ACDH	185.0	>4000.0	13.0	44.0
(CH ₂) ₄ NHCSNHMe	S-ACDH	>10000.0	110.0	45.0	15.0
(S)-CH ₂ - (4-imidazole)	R-ACDH	>10000.0	>4000.0	>10000.0	850.0
(S)-CH ₂ -[4-(2-aminothiazole)]	R-ACDH	>10000.0	>4000.0	208.0	300.0
H	R-ACDH	>10000.0	>4000.0	>10000.0	>10000.0

Table 6. Inhibition of release of the dipeptide Gly-Phe from the substrate Gly-Phe-Leu-Gly-Phe-Leu by cathepsin D in the presence of its D-isomers [91].

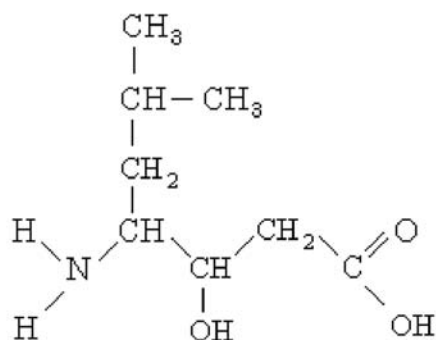
D-isomer	Hydrolysis, %	Inhibition, %
Gly-Phe-Leu-Gly-Phe-Leu	100.0	0.0
Gly-Phe-Leu-Gly-Phe-D-Leu	6.0	89.2
Gly-Phe-Leu-Gly-D-Phe-Leu	3.0	73.4
Gly-D-Phe-Leu-Gly-Phe-Leu	0.0	63.1
Gly-D-Phe-Leu-Gly-D-Phe-Leu	0.0	0.0
Gly-Phe-Leu-Gly-Phe-Leu []	0.0	0.0

up of L-amino acids. Cathepsin D vulnerability to hydrolysis and degree of inhibition by these analogues depend on the number and location of D-amino acids in the molecule (Table 6). The analogues with one D-amino acid at a maximum distance from the site sensitive to cathepsin D exert the strongest inhibitory effect. However, the analogues with two D-amino acids at the site of a cleaved Phe³-Leu³

bond and constituting a cyclic hexapeptide are not hydrolyzed and do not exhibit an inhibitory effect. This has been also confirmed by data shown in Table 7. Cathepsin D activity is also inhibited by Gly-Glu-Gly-Phe-Leu-Gly-D-Phe-Leu and aldehydes of such peptides as Ac-Leu-Leu-Nle-H, Ac-Leu-Val-Phe-H and [(s)-1-carboxy-2-phenyl-ethyl]-carbamoil-Arg-Val-Arg-H [93].

Table 7. Inhibition of cathepsin D and pepsin action by synthetic peptides [92].

Inhibitor	K _i μmol/l	
	Cathepsin D	Pepsin
pGlu-D-Phe-Pro-Phe-Phe-Val-D-Leu	0.031	0.470
D-Phe-Pro-Phe-Phe-Val-D-Leu	0.520	5.200
D-Phe-Pro-D-Phe-D-Phe-Val-D-Leu	650.000	no data
D-Phe-Pro-Phe-Phe-Val	no inhibition	no inhibition

**Fig. 4.** Statin.

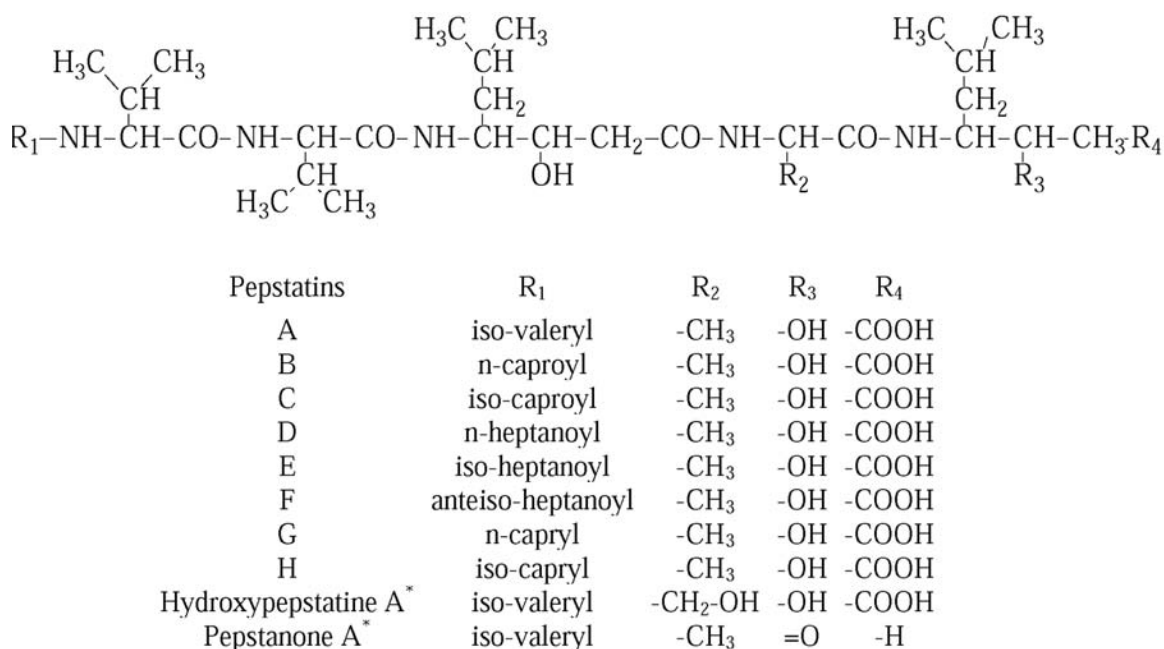
Inhibitors produced by microorganisms

Inhibitors of aspartyl proteases are synthesized by *Streptomyces testaceus*, *Streptomyces argenteolus* and other species of the genus *Streptomyces*. These inhibitors are called pepstatins as they inhibit the activity of pepsin. They also inhibit the activities of cathepsin D, cathepsin E, renin, pseudorenin, aspartyl proteases produced by microorganisms and plants [94-98].

Pepstatins are pentapeptides containing non-amino acid constituents, typical amino acids and atypical amino acid. They are composed of a sequence of 1 acyl radical, 2 valin residues, 1 statin residue, 1 alanine residue and 1 statin residue, and are shortly called Ac-L-Val-L-Val-L-Sta-L-Ala-L-Sta. Statin (Sta) is a rare atypical amino acid referred to as [(4S,3S)-4-amino-3-hydroxy-6-methylheptanoic acid] (AHMHA) (Fig. 4). Pepstatins differ from each other in the structure of the

acyl radical (Fig. 5). Hydroxypepstatin and pepstanon are pepstatin derivatives with preserved inhibitory potential. Hydroxypepstatin contains serine instead of alanine. In pepstanon, at the site of C-terminal Sta, its ketone 3S-3-amino-5-methylhexanone-2 (AMHN) is located. The isovalerian acid residue is the most common acyl, hydroxypepstatin and pepstanon residue (Fig. 6). The structure of acyl residue of pepstatins depends upon culture conditions. *Streptomyces testaceus* grown in a medium containing meat extract generates isovaleryl pepstatin, whereas that cultured in a medium with casein produces hexanol-pepstatin.

The hydroxyl group Sta4 is directly involved in the inhibitory action of pepstatins, reacting with the catalytic-site residues of an aspartyl protease [102]. The Sta4 residue is a structural analogue of substrate, analogue of its transitory state and shows an inhibitory action due to structural similarity to the tetraedric immediate product

**Fig. 5.** Structure of pepstatins, hydroxypepstatin and pepstanones. *- acyl radical R₁- the same as pepstatins A-H [100].

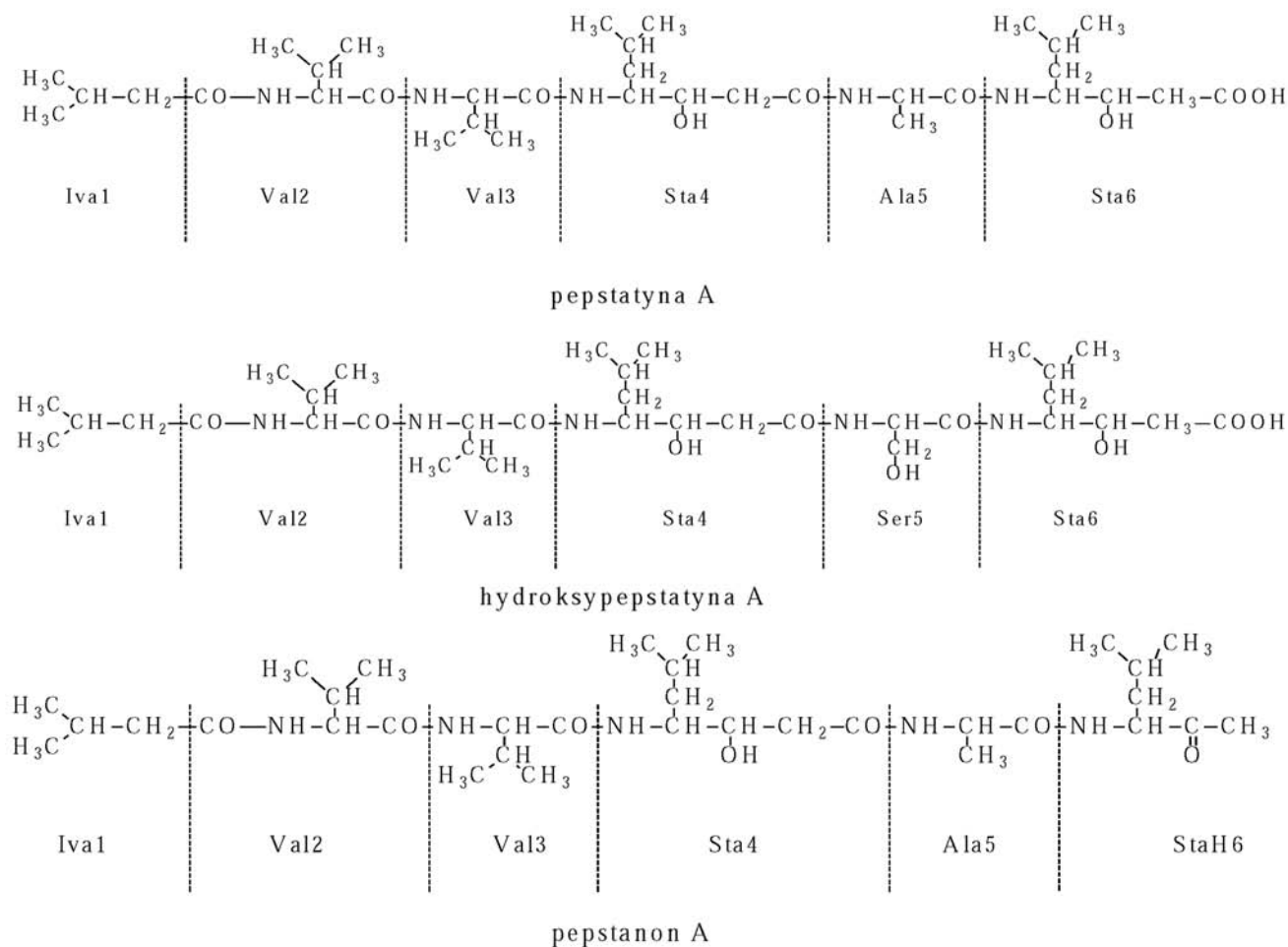


Fig. 6. Pepstatin A, hydroksypepstatin A and pepstanone A [101].

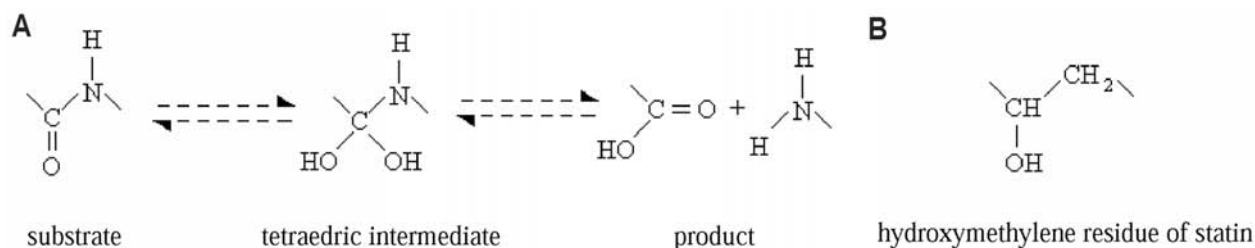


Fig. 7. Tetrahedric intermediate state of the substrate (A) and stable analogue of intermediate tetrahedric state which is hydroxymethylene residue (B) [103].

formed during cleavage of peptide bonds by proteases possessing carboxyl catalytic sites (Fig. 7). The mechanism of cathepsin D activity inhibition by pepstatin can be illustrated by a simplified model (Fig. 8) and a spatial model of these substances (Fig. 9). Hydrogen bonds connecting pepstatin to cathepsin D have been listed in Table 8. Pepstatin also binds to procathepsin D and inhibits its autoactivation [106].

The inhibition of aspartyl protease action by pepstatins depends to a large extent on the presence of acid residue in their structure [107]. A comparison between the inhibitory effect of pepstatins with vari-

ously structured acid residue on the activities of pepsin, cathepsin D and rennin shows that the inhibitory effects are similar in the case of the first two proteases, whereas that of rennin increases with elongation of carbon chain of the residue (Table 9). This is due to the structural similarity of the catalytic site of pepsin and cathepsin D and its different structure in the rennin molecule. Differences in the inhibitory action are also observed according to the type of the N- and C-terminal group (Table 10). The inhibitory effect of pepstatins on cathepsin D does not depend on the carbon chain length of the acid radical. Hydroksypepstatin

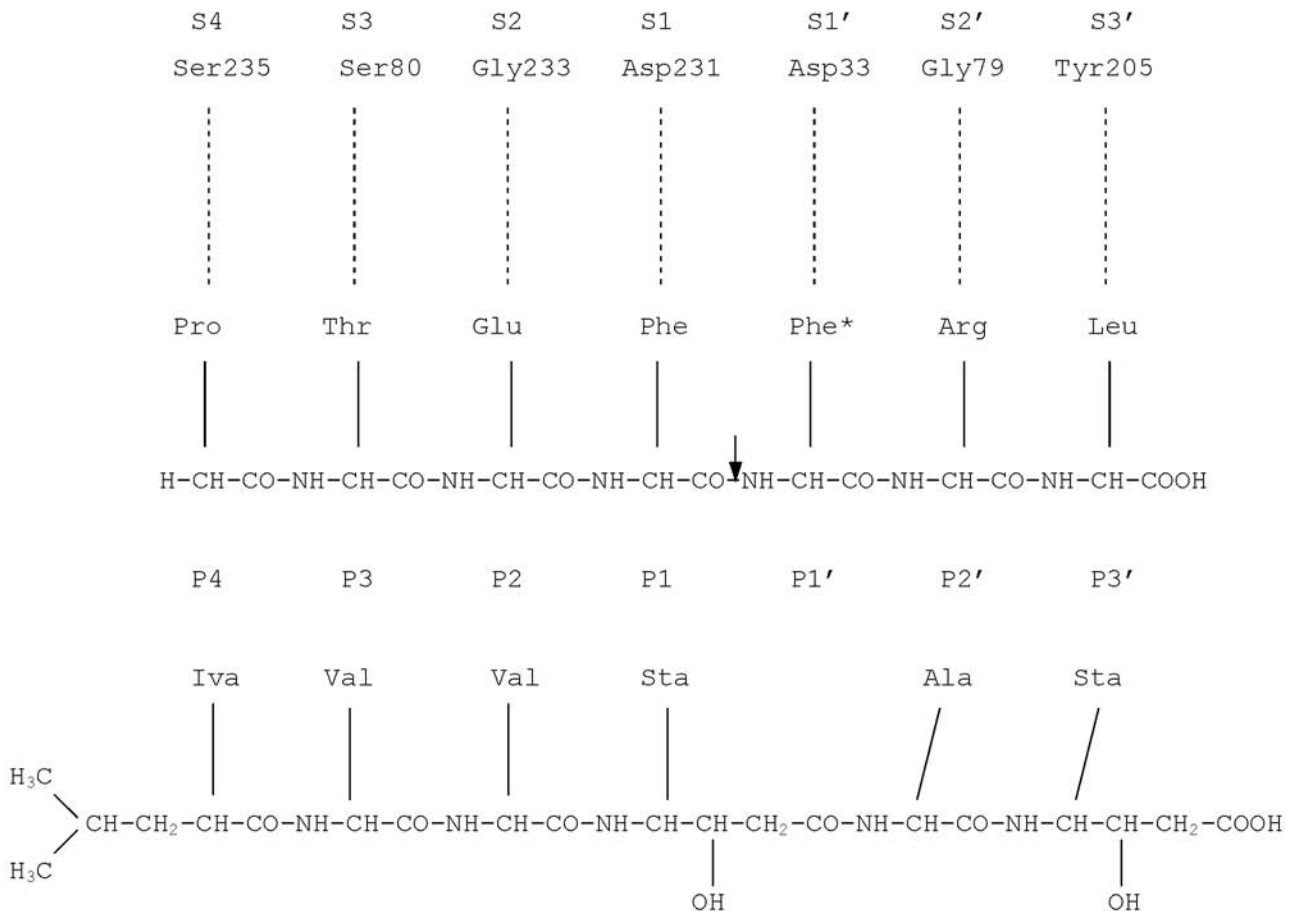


Fig. 8. Cleavage of a peptide bond -Phe4-Phe(NO₂)5 by cathepsin D in a substrate and pepstatin inhibition of this process, according to Schechter and Berger [104]. S4-S3' - aminoacidic residues of catalytic site, P4-P3' - substrate or pepstatin aminoacidic residues, arrow - cleaved peptide bond, * - NO₂.

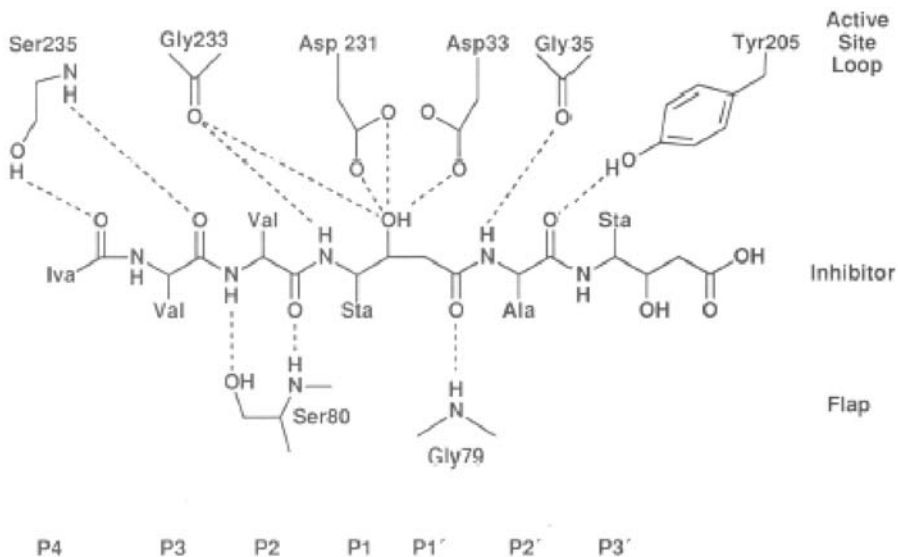


Fig. 9. Interactions between pepstatin and cathepsin D. Schematic hydrogen-bonding diagram for pepstatin bound to cathepsin D. Inhibitor side chain numbering as according to the nomenclature of Schechter and Berger, where Pn designates inhibitor side chain residues that interact with the corresponding S_n subsites within the enzyme active site. Iva and Sta indicate isovaline and statin, respectively [102,105].

exerts a similar inhibitory effect to that of pepstatins. However, the inhibitory effect of pepstanon is less pronounced. This indicates the significance of the presence of hydroxyl groups in the C-terminal fragment for the action of these inhibitors (Table 11).

The activity of cathepsin D is also inhibited by methyl ester of carbobenzoxy Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys [110].

The splitting off of the Ac1 and Val2 residues by pepstatin peptidase synthesized by *Bacillus Sphericus*

Table 8. Hydrogen bonds between cathepsin D and pepstatin; * aminoacid residues of the active site [102].

Cathepsin D		Pepstatin		Hydrogen bond [Å]
Residue	Atom	Residue	Atom	
Ser235	O γ	Iva1	O	2.6
Ser235	N	Val2	O	3.1
Ser80	O γ	Val2	N	3.5
Ser80	N	Val3	O	2.9
Gly233	O	Sta4	N	2.9
Gly233	O	Sta4	OH	3.4
Asp231*	O δ 1	Sta4	OH	2.8
Asp231*	O δ 2	Sta4	OH	2.6
Asp33*	O δ 1	Sta4	OH	3.5
Asp33*	O δ 2	Sta4	OH	2.8
Gly79	N	Sta4	O	2.9
Gly35	O	Ala5	N	2.9
Tyr205	OH	Ala5	O	2.9

inactivates pepstatins (Table 12). The action of this enzyme is conditioned by the N-terminal sequence Ac1-Val2-Val3-, from which acyl radical and N-termi-

Table 9. Inhibition of cathepsin D, pepsin and renin by pepstatins [101].

Pepstatin	IC ₅₀ μ mol/l		
	Cathepsin D	Pepsin	Renin
Acetyl	9.3	1.5	24.9
Propionyl	9.1	1.5	15.2
Butyryl	8.9	1.4	9.7
<i>iso</i> -Valeryl	8.8	1.4	6.6
<i>n</i> -Caproyl	9.3	1.4	4.3
<i>iso</i> -Heptanoyl	9.1	1.4	2.5
<i>n</i> -Capryl	8.9	1.4	1.7
<i>iso</i> -Valeryl hydroxyepstatin	11.4	1.7	20.0
<i>iso</i> -Valeryl pepstanone	17.0	2.0	39.0

nal valin are released. The splitting of the acyl and valin residues off pepstatin A causes a considerable reduction in the inhibitory activity towards cathepsin D, pepsin and renin (Table 13). Addition of the acyl residue, varying according to its type, increases the inhibitory activity.

Methods in combinatorial chemistry are used in studies on modification and synthesis of new pepstatin derivatives [113-116].

Table 10. Inhibition of cathepsin D, pepsin and renin activity by pepstatin and pepstatin derivatives [108].

Inhibitor	K _i μ mol/l		
	Cathepsin D	Pepsin	Renin
Iva-Val-Val-Sta-Ala-Sta	0.5	0.05	13000.0
Iva-His-Pro-Phe-His-Sta-Leu-Phe-NH ₂	210.0	27.0	19.0
His-Pro-Phe-His-Sta-Leu-Phe-NH ₂	900.0	40.0	50.0
POA-His-Sta-Leu-Phe-OCH ₃	3300.0	67.0	6300.0
POA-Leu-Sta-Leu-Phe-OCH ₃	35.0	12.0	27000.0
Iva-His-Pro-Phe-His-Sta-Ile-Phe-NH ₂	134.0	43.0	1.9

Table 11. Peptide aldehydes with inhibitory activity against cathepsin D [109]. Comparison with pepstatin.

Peptide aldehyde	IC ₅₀ . μ mol/l		
	Cathepsin D	Pepsin	HIV protease
Ac-Leu-Leu-Nle-H	35.0	24.0	5.2
Cbz-Ile-Phe-H	20.0	>200.0	4.8
Me-CO-Val-Phe-H	40.0	>200.0	5.4
N-Ac-Leu-Val-Phe-H	37.0	100.0	0.9
Iva-Val-Val-Sta-Ala-Sta-OH	<0.04	<0.005	2.0
Iva-Val-Val-Sta-Ala-Sta-H	17.0	2.0	3.0

Table 12. Substrate specificity of pepstatin hydrolase [111].

Substrates	Relative activity [%]	Ninhydrin-positive products
<i>iso</i> -Valeryl-Val-Val-AHMHA-Ala-AHMHA (pepstatin A)	100	Val, Val-AHMHA-Ala-AHMHA
<i>n</i> -Caproyl-Val-Val-AHMHA-Ala-AHMHA (pepstatin B)	723	Val, Val-AHMHA-Ala-AHMHA
Acetyl-Val-Val-AHMHA-Ala-AHMHA (pepstatin Ac)	250	Val, Val-AHMHA-Ala-AHMHA
<i>iso</i> -Valeryl-Val-Val-AHMHA-Ser-AHMHA (hydroxy-pepstatin A)	55	Val, Val-AHMHA-Ser-AHMHA
<i>iso</i> -Valeryl-Val-Val-AHMHA-Ala-3-amino-5-methylhexanone-2	2	Val, Val-AHMHA-Ala-3-amino-5-methylhexanone-2
Acetyl-Val-AHMHA-Ala-AHMHA	0	None
<i>iso</i> -Valeryl-Val-AHMHA-Ala-AHMHA	0	None
Benzoyl-Val-AHMHA-Ala-AHMHA	0	None
Phenoxyacetyl-Val-AHMHA-Ala-AHMHA	0	None
2-Phenoxypropionyl-Val-AHMHA-Ala-AHMHA	0	None
<i>iso</i> -Valeryl-Val	0	None
<i>iso</i> -Valeryl-Val-Val	0	None
<i>iso</i> -Valeryl-Val-Val-AHMHA	55	Val, Val-AHMHA
<i>iso</i> -Valeryl-Val-Val-AHMHA-Ala-AHMHA	100	Val, Val-AHMHA-Ala-AHMHA
Val-Val-AHMHA		Val, Val-AHMHA

Table 13. Inhibitory activities of Val-AHMHA-Ala-AHMHA, its N-acylated derivatives and pepstatin A against cathepsin D, pepsin and renin [112].

Compounds	ID ₅₀ µg/ml		
	Cathepsin D	Pepsin	Renin
Val-AHMHA-Ala-AHMHA	6.5	10.0	>250
Acetyl-Val-AHMHA-Ala-AHMHA	0.42	0.031	>250
<i>iso</i> -Butyryl-Val-AHMHA-Ala-AHMHA	0.28	0.021	>250
<i>iso</i> -Valeryl-Val-AHMHA-Ala-AHMHA	0.05	0.01	>250
Palmitoyl-Val-AHMHA-Ala-AHMHA	1.1	0.45	>250
Benzoyl-Val-AHMHA-Ala-AHMHA	0.05	0.031	>250
Phenoxyacetyl-Val-AHMHA-Ala-AHMHA	0.008	0.02	31
2-Phenoxypropionyl-Val-AHMHA-Ala-AHMHA	0.01	0.02	-
<i>iso</i> -Valeryl-Val-Val-AHMHA-Ala-AHMHA (pepstatin A)	0.011	0.01	4.5

Polypeptide plant inhibitors

Polipeptide inhibitors of aspartyl proteases can be found in spare plant organs such as seeds, bulbs and fruits, and more seldom in vegetative organs like leaves, roots and flowers. They protect plant proteins against uncontrolled actions of endogenous and exogenous proteases synthesized by parasitic viruses, bacteria, moulds and insects [117-121].

Well defined amino acid residues constituting the inhibitory reactive site, complementary to the amino acid residues of the protease catalytic site, are found on the surface of the polypeptide inhibitor molecule. The type of amino acid residues of this site determines

the group and individual specificity of the inhibitor. This can be a sequence specific to the catalytic sites of aspartyl proteases or to the catalytic site of a particular protease, also cathepsin D. The inhibitor forms a permanent complex with an in the 1:1 ratio and blocks the action of cathepsin due to hydrophobic and ionic hydrogen bonds of the reactive site of the inhibitor, the catalytic site of cathepsin and their closest environment.

Potato inhibitors of cathepsin D

Potato bulbs contain six or eight polypeptide inhibitors of cathepsin D, depending on the species [105,122-

Potato1	MMKCLFLLCLCLLPIVVSSTFTSQNLIDLPSSESPVKPVLDTNGKELNPSSYRIISIG	60
Potato2	MMKCLFFLCLCLFPIVVSSTFTSQNPINLPSSESPVKPVLDTNGKKNPNSSYRIISTF	60
Potato3	MMKCLFLLCLCLVPIVVSSTFTSQNPIDLPSSESPVKPVLDTNGKELNPSSYRIISIG	60
Potato4	-----ESPLPKPVLDTNGKELNPSSYRIISIG	28
Potato5	MMKCLFLLCLCLLPIVVSSTFTSQNPIDLPSSESPVKPVLDTNGKELNPSSYRIISIG	60
Potato6	-----NSSYRIISIG	10
Potato7	-----NSSYRIISIG	10
Potato8	-----ESPLPKPVLDTNGKELNPSSYRIISIG	28

Potato1	RGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSTNIFEDQLLNIQFNIP	120
Potato2	WGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSTNIFEDQLLNIQFNIP	120
Potato3	RGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSGGIFEDQLLNIQFNIP	120
Potato4	RGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSGGIFEDQLLNIQFNIA	88
Potato5	RGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTFVRFIPLSGGIFEDQLLNIQFNIA	120
Potato6	RGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSGGIFEDQLLNIQFNIP	70
Potato7	RGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSTNIFEDQLLNIQFNIP	70
Potato8	AGALGGDVYLGKSPNSDAPCPDGVFRYNSDVGPSGTPVRFIPLSGGIFEDQLLNIQFNIP	88
	*****.***** ***** .*****	
Potato1	TVKLCVSYTIWKVGNLNAHLRMTLLETGGTIGQADSSYFKIVKSSKFGYNLLYCPITR-H	179
Potato2	TVKLCVSYTIWKVGNLNTHLWMTLLETGGTIGKADSSYFKIVKSSKFGYNLLYCPITRPP	180
Potato3	TVRLCVSYTIWKVG-INAYLRTMLETGGTIGQADSSYFKIVKSSILGYNLLYCPITR-P	178
Potato4	TVKLCVSYTIWKVGNLNAYFRMTLLETGGTIGQADSSYFKIVKLSNFGYNLLYCPITP-P	147
Potato5	TVKLCVSYTIWKVGNLNAYFRMTLLETGGTIGQADSSYFKIVKLSNFGYNLLYCPITP-P	179
Potato6	TVKLCVSYTIWKVGNLNAYFRMTLLETGGTIGQADNSYFKIVKSSKIGYNLLSCLPFTS--	128
Potato7	TVKLCVSYTIWKVGNLNAYFRMTLLETGGTIGQADNSYFKIVKSSKIGYNLLSCLPFTS--	128
Potato8	TVKLCVSYTIWKVGNLNAYFRMTLLETGGTIGQADNSYFKIVKLSNFGYNLLSCLPFTS--	146
	:::***** :*:: *****:*.***** * :***** **:	
Potato1	FLCPFCRDDNFCAKVGVIQNGKRRRLALVNENPLDVLVFQEV	220
Potato2	IVCPFCRDDDFCAKVGVIQNGKRRRLALVNENPLDVLVFQEV	221
Potato3	ILCPFCRDDDFCAKVGVIQKGRRLALVNENPLDVNFKEV	219
Potato4	FLCPFCRDDNFCAKVGVIQNGKRRRLALVNENPLDVLVFQEV	188
Potato5	FLCPFCRDDNFCAKVGVIQNGKRRRLALVNENPLDVLVFQEV	220
Potato6	IICLRCPEDQFCAKVGVIQNGKRRRLALVNENPLDVLVFQE-	168
Potato7	IICLRCPEDQFCAKVGVIQNGKRRRLALVNENPLDVLVFQEV	169
Potato8	IICLRCPEDQFCAKVGVIQNGKRRRLALVNENPL-----	180
	::* * :*:*****:*****	

Fig. 10. Amino acid sequences of aspartic protease inhibitor from potato. * - identical amino acid residues; :: - conserved amino acid residues [132].

131]. They show a considerable similarity in the amino acid composition, sequence (Fig. 10) and in the number and location of disulphide bridges. Potato inhibitors possess two inhibitory sites: one binding cathepsin D and the other binding trypsin [133], and they do not inhibit the activity of pepsin. Inhibitors of aspartyl proteases also occur in potato leaves [134].

Inhibitors from pumpkin fruit

Cathepsin D inhibitor, which also inhibits the activity of pepsin and acid protease from fungus *Glomerella cingulata* has been isolated from pumpkin fruit [135]. It is composed of 96 amino acid residues and occurs in three molecular forms differing slightly in the amino

acid sequence. Two molecular isoforms are found to predominate (Table 14) and are called DNIS and HDVA due to differences in the amino acid sequence (Fig. 11). The sequence, however, is very similar, especially in the N-terminal sequence (MGPGAIGEVIG) and in the internal sequence (FYNVVVLEK). These inhibitors do not contain cysteinyl residues or sugar constituent. The molecular mass of DNIS isoform is 10551 Da, and of HDVA isoform - 10527 Da. These isoforms constitute a dimeric form with a molecular mass of 21000 (10551 + 10527). During purification, the dimeric form breaks down and monomers preserve their activity. The inhibitor is permanent in an acid and neutral environment, but sensitive to alkalization and elevated temperature.

Table 14. Aminoacidic composition of DNIS and HDVA inhibitors isolated from pumpkin fruit [135].

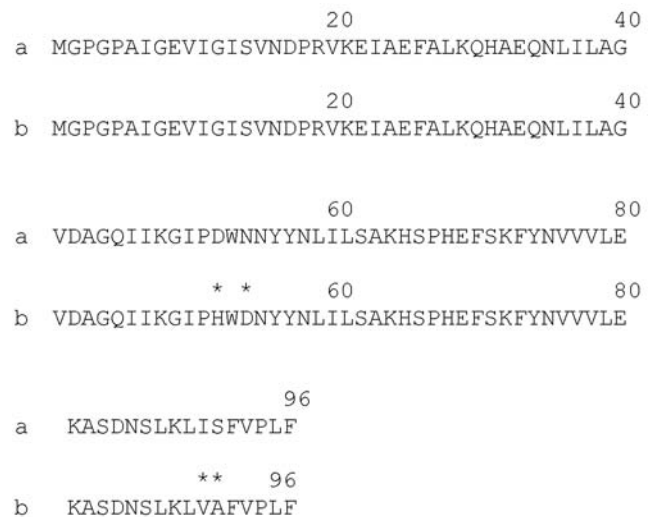
Aminoacid	DNIS	HDVA
S	7	6
G	7	7
H	3	4
R	1	1
T	0	0
A	8	9
P	6	6
Y	3	3
V	8	9
M	1	1
I	10	9
L	9	9
F	5	5
K	7	7
W	1	1

Inhibitors synthesized by other plants

Cathepsin D inhibitors can be found in the seeds of lentil and vicia [136], but are not found in the seeds of 25 cereal species and pulses consumed by man. Cathepsin D inhibitors have been isolated from the seeds of wheat [137], barley [138], tomato leaves [139-141], tobacco leaves [142], aubergine fruit

Table 15. Inhibitor isolated from tomato leaves and inhibitory effect on different aspartyl proteases [143]. All measurements were performed in pH=4.7.

Aspartyl proteinase	Ki, nmol/l*
Bovine chymosine	>1200
Cyprosin	>1200
Endotiapepsin	>2400
Human gastricsin	>1200
Cardosin B	>2400
Human cathepsin D	260
Human cathepsin E	>1200
Penicyllopepsin	>1200
Human pepsin	>2400
Yeast proteinase A	30
Proteinase HIV-1	>1000

**Fig. 11.** Amino acid sequences of DNIS (a) and HDVA (b) isoforms of squash aspartic proteinase inhibitor [135].

(*Solanum melongea*) and from trailing nightshade leaves (*Solanum dulcamara*) and black nightshade (*Solanum nigrum*). The inhibitor obtained from tomato leaves inhibits the activity of the proteases listed in Table 15. The effects of cathepsin D inhibitors isolated from tomato leaves and potato bulbs have been compared in Table 16.

The synthesis of plant cathepsin D inhibitors is stimulated by mechanical tissue injury [144], as well as jasmonic acid, methyl ester of jasmonic acid and abscisic acid [145-149]. Salicylic acid inhibits synthesis of these inhibitors [150].

Techniques of molecular biology have been used to obtain plant cathepsin D inhibitors [146,151,152].

Inhibitors synthesized by lower animals

Cathepsin D activity is inhibited by equistatin, a protein inhibitor isolated from *Actinia equina* [153,154]. Equistatin inhibits the activity of cysteine proteases: papain, cathepsin B, cathepsin L and cathepsin D, but not the activity of pepsin, chymotrypsin or HIV protease. Equistatin has a domain structure (Fig. 12).

Table 16. Inhibition of cathepsin D activity indifferent pH by inhibitor isolated from tomato leaves and potato bulbs [143].

Inhibitor	pH	Cathepsin D Ki, nmol/l
Isolated from potato	3.1	30.0 ± 5.0
	4.7	5.0 ± 0.5
Isolated from tomato	3.1	580.0 ± 100.0
	4.7	260.0 ± 30.0

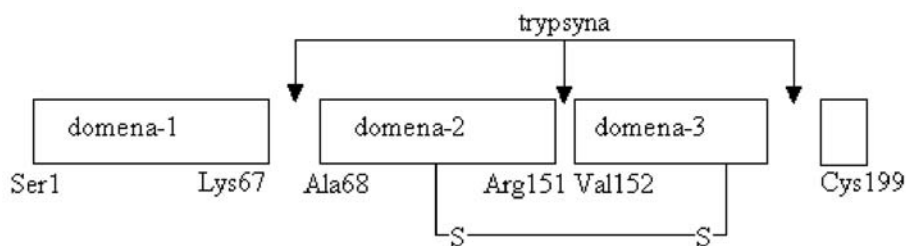


Fig. 12. Schematic diagram of equistatin [154].

Table 17. Inhibition constants (Ki) of aspartyl proteases and papain with domain-1, domain-2, 3 and equistatin [154].

Protease	Inhibitor	Ki, nmol/l
Cathepsin D	equistatin	0.30
	domain-1	>1000.00
	domain-2, 3	0,40
Pepsin	equistatin	>1000.00
Chymosine	equistatin	>1000.00
HIV protease	equistatin	>1000.00
Papain	equistatin	0.57
	domain-1	0.61
	domain-2, 3	>1000.00

Table 18. Kinetic constants (Ki) for the inhibition of aspartic proteases by the *Ascaris suum* inhibitor [207].

Protease	pH	Ki, nmol/l
Pepsin	4.0	2.6
Cathepsin E	3.0	3.4
Cathepsin D	4.0	>10 ⁴
Renina	6.0	>10 ⁴

Trypsin digestion of equistatin leads to the formation of domain-1 and domain-2 and 3, which are connected by the disulphate bridge. These domains originate from cleavage of the peptide bonds Lys67-Ala68 and Arg151-Val152. Trypsin also splits the inactive C-terminal fragment off domain-3. Domain-1 inhibits the activity of cystein proteases (papains), whereas domain-2 and domain-3 inhibit the activity of cathepsin D (Table 17).

Ascaris lumbricoides and *Ascaris suum* synthesize the inhibitors which block the activity of cathepsin E, pepsin and rennin but not cathepsin D [155-158]. They are used to differentiate the activities of these proteases in the nonfractionated material. The activity of the inhibitor from *Ascaris suum* towards aspartyl proteases has been presented in Table 18.

Endogenous cathepsin D inhibitors

Endogenous cathepsin D inhibitors include α 2-macroglobulin and polyanion compounds - glycozaminoglycans and deoxyribonucleic acids.

The α 2-macroglobulin (α 2-M), a 725,000-Da glycoprotein, is built up of four structurally, electrophoretically and immunologically identical 185,000-Da subunits. Each subunit is composed of 1,451 amino acid residues and contains 8 oligosaccharide chains. The subunits combine to form dimers by

means of disulphide bonds Cys225-Cys408 and Cys-447-Cys540. The dimers attached to hydrogen- and hydrophobic-bonds form a tetramer. The α 2-M differs from typical inhibitors in protease binding and low specificity, and it shows a two-stage interaction with proteases. In the first stage, protease recognizes a specific peptide bond in the central fragment of the polypeptide chain of each subunit with approximately 45 amino acid enzymes [160,161]. In this fragment, peptide bonds formed by residues of 15 amino acids meet the specificity requirements of each endopeptidase ("bait" principle) [162]. Therefore, the α 2-M is a universal broad-spectrum inhibitor, reducing the activity of all four endoprotease classes [163-165]. In the case of cathepsin D, the -Phe684-Tyr685- bond undergoes cleavage. However, the α 2-M exerts no effect on the activity of exopeptidases, as the peptide bond situated within the chain cannot be cleaved by aminopeptidases, carboxypeptidases, tripeptidases and dipeptidases. Specific peptide bond cleavage causes a rapid change in the conformation of all subunits and protease arrest in stage two ("trap" principle). Thus, protease becomes inaccessible to protein substrates and macromolecular inhibitors. Immediate conformational changes in both α 2-M subunit pairs block the binding of the second protease molecule. It means that only one α 2-M molecule binds only one protease molecule. The other protease molecule can be bound by α 2-M following cleavage of the peptide bond in the second pair of subunits, prior to conformational changes initiated by the first molecule. Then, the two protease molecules are bound at two independent but equivalent sites of the α 2-M molecule, which may occur only at a high concentration of a relatively low molecular mass protease, e.g. trypsin or chymotrypsin, but not in the case

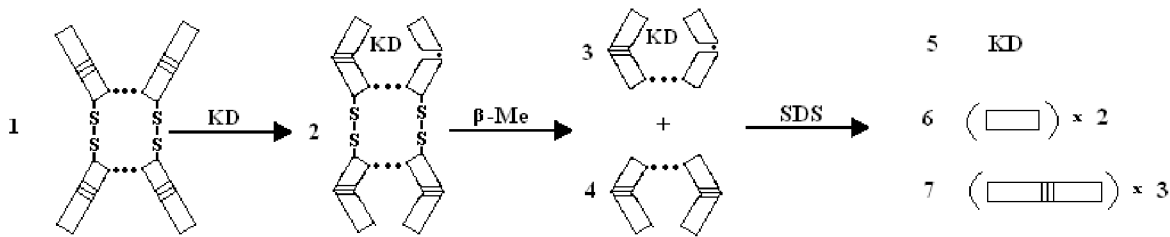


Fig. 13. Schematic structure of α_2 -macroglobulin binding with cathepsin D (KD) and action of β -mercaptoethanol (β -Me) and dodecyl sulphate (SDS) on inhibitor-cathepsin D complex. \equiv - "bait" region, 1 - tetramer, 2 - tetramer-cathepsin D complex, 3 - dimer-cathepsin D, 4 - dimer, 5 - cathepsin D, 6 - fragments of monomer, 7 - monomers.

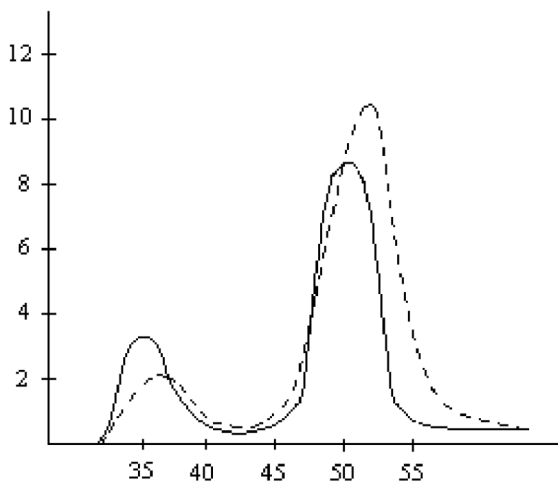


Fig. 14. Gel chromatography of ^{125}I -labelled cathepsin D [169]. Cathepsin D and α_2 -macroglobulin - solid line, cathepsin D with pepstatin and α_2 -macroglobulin - stroked line.

of cathepsin D that occurs in low concentrations in body fluids and possesses a relatively high molecular mass. The reaction of α_2 -M with cathepsin D takes place according to the following scheme: $\alpha_2\text{-M} + \text{KD} \rightarrow \alpha_2\text{-Mx} + \text{KD} \rightarrow \alpha_2\text{-Mx-KD}$. In physiological conditions, cathepsin D binding to α_2 -M is irreversible. The use of depolymerizing chemical compounds allows observation of the course of cathepsin D-induced changes in the structure of α_2 -M subunits (Fig. 13). β -mercaptoethanol (1%) cleaves tetramers to form a complete dimer and a dimer built up of a full dimer and one half of the monomer whose other half has been released. Dodecylsulphate (0.1%) releases noncovalently bound complete and incomplete monomers. The catalytic site of cathepsin D, occupied by α_2 -M, is free. Micromolecular substrates and micromolecular inhibitors have access to this site, whereas the macromolecular ones do not [166]. The α_2 -M inhibits the residual activity of cathepsin D at pH=6.0-6.2, but not at a lower pH, because of structural instability of this protease [167,168]. Approximately 20% of plasma cathepsin D is blocked by α_2 -M [169]. The remaining 80% is the procathepsin D, the inactive precursor which does not interact with α_2 -M. The

above data were obtained by means of Sephadex G-75 gel chromatography using ^{125}J -labelled cathepsin D (Fig. 14). Pepstatin blocks the interaction between cathepsin D and α_2 -macroglobulin, due to which the amount of cathepsin D bound to the inhibitor is reduced to approximately 8%. The α_2 -M is synthesized in fibroblasts and then passes to the intracellular fluid, lymph and blood [170]. In the plasma, α_2 -M occurs at a concentration of 260.0 mg/100 ml (3.3 $\mu\text{mol/l}$), constituting approximately 0.4% of plasma proteins [171]. The physiological role of α_2 -M is to prevent proteins against uncontrolled proteolysis, which takes place through binding, activity inhibition and removal of proteases from blood plasma and intracellular fluid. The α_2 -M-protease complexes are captured by membrane receptors of macrophages and hepatocytes [172], undergo endocytosis and are transported to lysosomes. Then, the complexes undergo digestion and the receptors return to the cell surface. The antiproteolytic effect of α_2 -M is counteracted by methylamine [173].

Cathepsin D reduces formation of ion complexes with glycozaminoglycans [174] and polysaccharides [98]. The strongest inhibitory effect is exerted by heparin (52%), weaker by chondroitin sulphate (36%) and the weakest by hialuron acid (10%). Reduction in cathepsin D activity by glycozaminoglycans is pH-dependent. The greatest decrease can be observed at pH=4.0-4.5, whereas at a lower pH, the inhibitory effect is reduced. The inhibitory effect of glycozaminoglycans is counteracted by alkaline proteins.

The activity of cathepsin D is also reduced by formation of ion complexes with 18b and 53b DNA fragments [73]. There is a correlation between the inhibitory activity of cathepsin D and the melting temperature (T_m) of a DNA molecule. These fragments also decrease the activity of cathepsin E, but not the activity of seryl and cysteine proteases.

Antibodies

Cathepsin D (antigen) introduced parenterally to another species induces production of antibodies (antienzymes). Antigen determinants and catalytic sites of

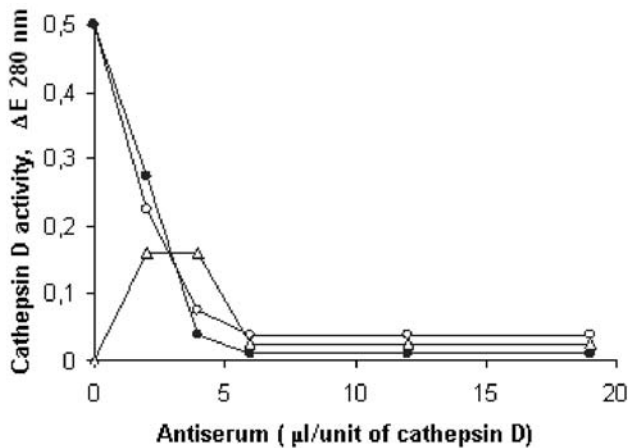


Fig. 15. Effect of antiserum ratio on cathepsin D activity in pH=5.5 [176]. o - before centrifugation, • - in supernatant, ▲ - in precipitate.

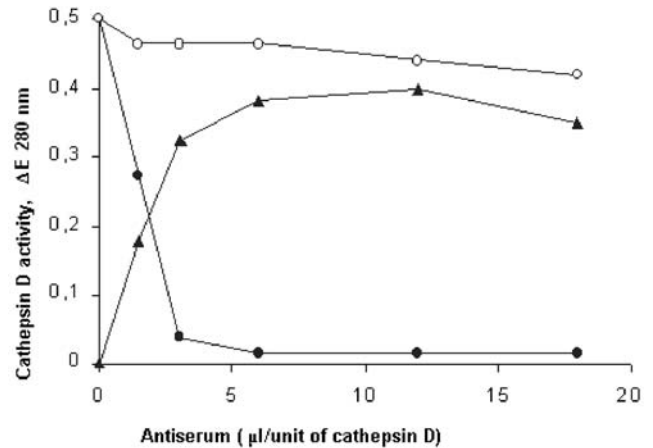


Fig. 16. Effect of antiserum ratio on cathepsin D activity in pH=3.2 [176]. o - before centrifugation, • - in supernatant, ▲ - in precipitate.

cathepsin D are located at adjacent sites in a molecule. The cathepsin D binding to an antibody hinders access of substrate to the catalytic site [175]. The site is larger for the macromolecular than it is for the micromolecular substrate. The inhibitory effect also depends upon the chemical structure of a substrate, being more pronounced for hemoglobin than for proteoglycans [176]. The cathepsin D has a few sequential and conformational antigenic determinants against which antibodies are produced. The inhibitory effect has been shown to be exerted by antisera and isolated antibodies. Anti-cathepsin D antibodies have been obtained from Sepharose 4B-bound cathepsin D using an immunosorption method. The inhibitory effect is also found to be exerted by monovalent fragments of antigen binding (Fab) of immunoglobulins, due to antibody fragmentation with papain or pepsin and distribution of the products obtained in Sephadex G-75 [177,178]. Antibodies are the most specific cathepsin D inhibitors. Their specificity is absolute, referring not only to cathepsin D but also to its species-related origin. Antibodies directed against cathepsin D isolated from a particular organ also inhibit the action of this cathepsin isolated from other organs of the same species, but do not react with cathepsin D from other species. Complete inhibition of cathepsin D activity occurs at an approximately six-fold molar excess of antibodies. The formation of the cathepsin D-antibody complexes is most intense at pH=5.0 [179]. It is then that cathepsin D solubility decreases and shedding from the solution takes place. The activity of cathepsin D measured at pH=5.0 with the use of hemoglobin is almost completely hampered in a reactive mixture prior to centrifugation, in supernatant and sediment (Fig. 15). However, the activity of cathepsin D is not inhibited by antiserum at pH=3.2 (Fig. 16). No activity can be found in the supernatant, whereas in the sed-

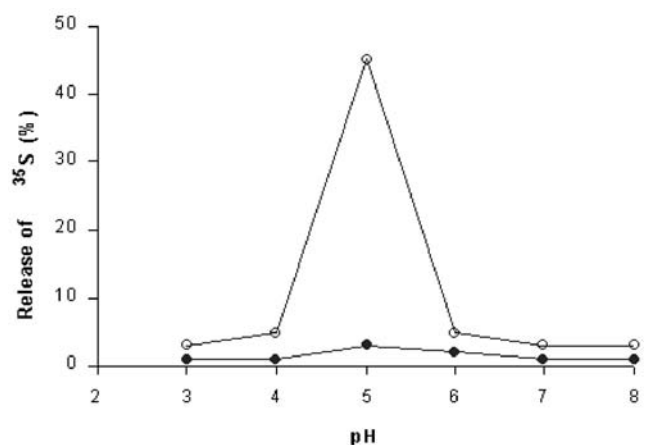


Fig. 17. Inhibitory effect of antiserum on cathepsin D activity against ³⁵S-proteoglycans. • - cathepsin D, ▲ - cathepsin D and antibody [176].

iment the activity is nearly the same as with noncentrifuged material. Thus, due to immunoinhibition, there is a shift in the optimum pH of cathepsin D to acid direction [176]. The effect of immunocomplex formation on the action of cathepsin D on proteoglycans has been shown in Fig. 17. Conformational changes in the cathepsin D complexed with antibody stabilize the structure and cause an increase in its thermoresistance. The antibodies against lysosomal proteases accumulate in cultured fibroblast lysosomes [180].

The biological role and application of cathepsin D inhibitors

The acid pH of lysosomes has a basic role in the activation of procathepsin D and activity of cathepsin D. The propeptide released during autoactivation of procathepsin D, which at weakly acid pH binds electro-

Table 19. Distribution of ^3H -pepstatin after enteral introduction in mice [201].

Organ	Time/ μg			
	2 h	6 h	12 h	24 h
Colon	63.732	71.338	1.479	0.037
Stomach	17.338	13.380	0.135	0.101
Jejunum	11.224	5.900	0.352	0.178
Urine	2.097	2.963	4.304	5.803
Skeleton	0.418	0.455	0.276	0.229
Skin	0.381	0.426	0.262	0.262
Liver	0.247	0.205	0.170	0.130
Muscles	0.244	0.316	0.170	0.039
Plasma	0.036	0.088	0.075	0.071
Erythrocytes	0.009	0.034	0.038	0.029
Faeces	0.0	3.754	84.879	77.178
Other organs	0.251	0.344	0.417	0.246
Summary	95.977 (96.5%)	99.203 (99.9%)	92.557 (93.0%)	84.303 (85.0%)

statically to cathepsin D and inhibits its activity, also plays a certain regulatory part [70,71]. In the case of lysosomal damage and cathepsin D translocation to the cytosol of the intercellular fluid and plasma, neutral pH and α 2-macroglobulin prevent uncontrolled proteolysis [181].

Exogenous cathepsin D inhibitors play a key role in identification of the structure of the catalytic site of this protease [57]. The binding of a radioisotope-labeled inhibitor to the catalytic site of cathepsin D, limited proteolysis and isolation of a fragment containing a marker allow determination of the composition and the amino acid sequence of the site. A comparison of differences in sensitivity to inhibitors facilitates differentiation of aspartyl proteases. The activities of cathepsin E and pepsin are found to be blocked by aspartyl protease inhibitor from human ascaris, whereas cathepsin D and renin are insensitive to this inhibitor.

Cathepsin D inhibitors have analytical and preparative significance. Pepstatin has been used for quantitative determination of this protease by catalytic site titration, whereas Dansyl-pepstatin for cathepsin D catalytic site titration [182]. Lysosomal protease inhibitors are added to the solutions used to obtain subcellular fractions and fractionation of cell proteins in order to prevent proteolytic degradation. High specificity of binding to inhibitors allows isolation of cathepsin D by means of affinity chromatography, using inhibitors [20,183-186] and constant carrier-bound antibodies. Differences in the pH values between procathepsin and

cathepsin D bonds with pepstatinyl-agarose help separate these two forms of the enzyme [187,188]. The constant carrier-bound cathepsin D is used to isolate its inhibitors [189]. The carrier-bound propeptide allows cathepsin isolation [190].

Pepstatin is used to determine the function of the cathepsin D found in a mixture with other cathepsins in protein degradation that occurs in the cell [191,192]. Inhibition of cell proliferation by pepstatin confirms the hypothesis that this process is stimulated by cathepsin D. The use of specific antibodies against cathepsin D has shown its significant involvement in degradation of macrophage proteins and cartilage proteoglycans [193]. Cathepsin D is localized within cells and tissues by means of specific fluorescein-labeled antibodies [20]. Methylmercury derivatives of pepstatin have been used for intracellular localization of cathepsin D [194]. The immunochemical method allows differentiation between cathepsin D and cathepsin E [195].

Attempts have been made to use cathepsin D inhibitors for therapeutic purposes. Synthetic inhibitors, due to their high toxicity, cannot be used for these purposes. Natural inhibitors can regulate proteolysis velocity in vivo. Most studies have dealt with the therapeutic application of pepstatin. Pepstatin has been used in pathological conditions occurring with increased cathepsin D activity, such as inflammatory states, burns, gastric ulcer, hypertension and Shwartzmann - Sanarelli phenomenon [99, 103, 132, 196-200]. Its advantage consists in low toxicity and low molecu-

lar weight, which permit cell and tissue penetration. Pepstatins can be administered orally or intraperitoneally. Pepstatin dose of DL_{50} is very high - in rats 2.0 g/kg of body weight for oral administration and 0.875 g/kg for intraperitoneal administration [200]. Pepstatin distribution in mouse organs has been presented in Table 19. Low toxicity of pepstatin allows it to be used to investigate processes dependent on cathepsin D effect on culture cells [202]. Anti-cathepsin D antibodies have been also used to inhibit proteolysis *in vivo*. Antibodies are obtained by cells via pinocytosis and accumulated in lysosomes. This causes inhibition of degradation of the hemoglobin added to a macrophage culture. Antibodies have no effect on cell viability or pinocytosis.

One inhibitor cannot inactivate all proteases of the lysosomal extract. Only a mixture of inhibitors, each inactivating protease of different catalytic site structure, may inhibit cell proteolysis [203]. A similar effect can be achieved thanks to polyvalent immune serum obtained after administration of the lysosomal extract to experimental animals. The immune serum added to a fibroblast culture was stored in lysosomes, which showed lower activity of cathepsins, especially cathepsin D and reduced exogenous protein degradation potential. Pinocytosis was impaired and lysosomes enlarged.

Determination of concentration, activity and tissue and cell location of cathepsin D inhibitors

The activity of cathepsin D is determined using a test, in which: 1 - enzyme releases the reaction product in an amount corresponding to the absorbance of 0.5; 2 - inhibitor inhibits 50% of enzyme activity. The inhibitor activity is calculated according to the formula: inhibitor, U/ml/min = $(d \times r) : t$, in which: d means difference in the amount of the reaction products in a test without and with inhibitor, r - inhibitor dilution, t - time of incubation. Determination of the effect of the preincubation time of protease with inhibitor on the measurement outcome allows detection of its immediate or progressive action. A synthetic cathepsin D substrate, namely Pro-Thr-Glu-Phe-Phe(NO_2)-Arg-Leu should be used for determinations [104]. The use of protein substrate (hemoglobin) is not recommended due to the fact that indirect degradation products undergo further digestion and final degradation products may inhibit protease activity. Measurement of the kinetic constants in the presence of many substrates whose concentrations are varied may be biased.

The inhibitory constant dimension (K_i), *i.e.* enzyme-inhibitor dissociation constant, is the measure of inhibitor activity; the lower the K_i value, the higher the affinity of inhibitor activity for the enzyme [204].

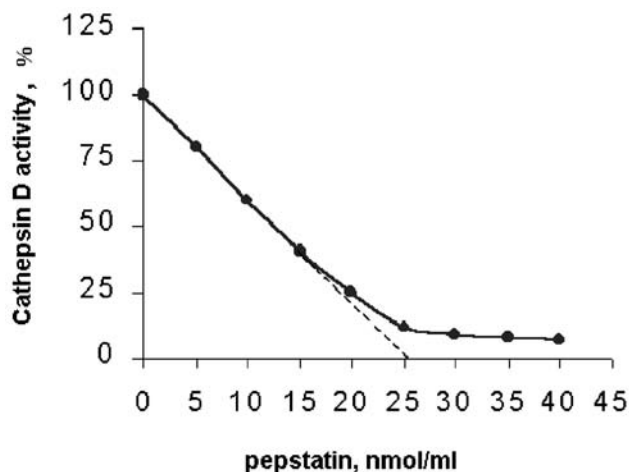


Fig. 18. Titration of the cathepsin D active site [207].

To compare the suppressive actions of various inhibitors, their concentrations that reduce cathepsin D activity by 50% (ID_{50}), expressed in μM , are determined [204]. The enzyme-inhibitor molar ratio is determined from a plot of cathepsin D activity correlation (in %) as a function of enzyme-inhibitor molar ratio [205,206] (Fig. 18). The curve bends at higher inhibitor concentrations due to partial dissociation of the complex. The inhibitor concentration that causes a 50% enzyme activity reduction is used for analysis. Prior to the therapeutic application of the inhibitor, its toxicity expressed by DL_{50} value should be determined. The activity of α_2 -M is measured through the assessment of proteolytic activity reduction in a standard testing system. The α_2 -M concentration is assayed, using antibodies, by the nephelometric method, laser nephelometry and radial immunodiffusion [208]. Cell and tissue α_2 -M localization is also performed [209].

Determination of activity and concentration of cathepsin D inhibitors in bacterial and plant extracts may appear biased as also proteases with acidic pH optima can be present in the material [210]. These proteases, either sensitive or insensitive to the inhibitors, reduce the value of inhibitor activity - inhibit the activity (sensitive) and cause proteolytic inactivation (insensitive). The outcome depends upon the pH of the extraction fluid and temperature and time of extract storage. In the case of stable micromolecular inhibitors, proteases are inactivated thermally and by means of trichloroacetic or perchloric acid. Trichloroacetic acid is removed from the supernatant, whereas perchloric acid is precipitated by means of potassium hydrogen and inhibitor activity is determined as above. The outcome can also be affected by the order in which reagents are added: inhibitor - enzyme - substrate or inhibitor - substrate - enzyme

[211]. The activity and concentration of inhibitors in the plasma and systemic fluids are calculated per unit of volume, in cell culture per cell count and in solid tissues per 1g or 1 μ g DNA.

Results of immunohistochemical assessment of inhibitor distribution (mainly α 2-M) in cells and tissues have been presented in color microphotographs. Reaction intensity is evaluated semiquantitatively with the scale: lack of reaction (-), weak positive reaction (\pm), positive reaction (+), strong positive reaction (++) [212, 213]. Cathepsin D is localized in tissues by means of biotin- and biman-labeled pepstatin [214, 215].

Conclusions

The use of inhibitors has broadened the knowledge of the structure of the catalytic site, the mechanism of action and contribution of cathepsin D to protein degradation in lysosomes. Attempts have been also made to apply the inhibitors in the therapy of diseases occurring with cathepsin D involvement. Especially cathepsin D inhibitors, including statin, play a role, due to their low toxicity. The application range is even wider as statin is also the basic component of certain inhibitors of HIV protease [216-222] and of many renin inhibitors used for the treatment of hypertension and atherosclerosis [108, 223-226].

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