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REVIEW ARTICLE

Introduction (p.1)

A number of proteinases or peptidases exist in various animal tissues (liver, kidney, spleen and etc.). Although the substrate specificity and physicochemical properties of these enzymes show a wide variation, all of these are intracellular enzymes which differ in many respects from extracellular enzymes such as trypsin, chymotrypsin and pepsin. Of these intracellular enzymes, a group of those which possess acidic optimum pH is known generically as "cathepsins".

I Distribution and Localization

Cathepsins are present in most organ or tissue extracts and are found in large quantities in liver, kidney, spleen and lung[1-3]. The richest source for extraction is the spleen. Although muscles, cerebral tissues and red blood cells are

known to contain a very small amount of cathepsins,[1] a certain type of cathepsin has been recently extracted from the brain[4]. Also, the presence of cathepsins has been demonstrated in cartilage tissues[5, 6].

Intracellular localization of cathepsins has been studied in liver tissue homogenates from rats by de Duve et al.[7] who showed that nearly all of cathepsin activity was found in the heavy and light mitochondrial fractions (so-called lysosome fraction) but none in the microsomal fraction or in the final supernatant.

II Classification

Cathepsins are classified into five types, A, B, C, D and E, according to their optimum pH, substrate specificity and degree of activation by cysteine. However, there are other types of cathepsins which do not appear to belong to either one of these five. Tallan, Jones and Fruton[8] first classified cathepsins, according to their specificity to synthetic peptide substrates, into A (pepsin-like), B (trypsin-like) and C (chymotrypsin-like). Later, the presence of other type of cathepsin that does not act on synthetic substrates was isolated[9-11] and this was named cathepsin D by Press et al.[10]. Lapresle and Webb[11] have described another type of non-synthetic substrate-hydrolyzing cathepsin, E. General properties of these five types of cathepsins are listed in the following table:

a)分類	b)至適 pH	c)cysteine 要求	d)基質
A	5.7	不要[1]	CBZ-GluTyr
B	5.3	必須[2]	Benzoyl Arg-NH_2
C	5.0~6.0	活性化[3]	GlyPhe-NH_2
D	3.5	不要[1]	Hb or Alb
E	2.5	不要[1]	Alb

a) classification
b) optimum pH
c) cysteine requirement
1) not necessary
2) essential
3) activated
d) substrate

*(注) ↙は分解する部位を指す.

* Arrow indicates the location of attack by the enzyme.

**略号 : CBZ-GluTyr : carbobenzoxy-L-glutamyl-L-tyrosine
Benzoyl Arg-NH_2 : benzoyl-L-argininamide
GlyPhe-NH_2 : glycyl-L-phenylalaninamide
Hb : hemoglobin Alb : albumin

** Glossary for abbreviations used.

III Determination of Enzyme Activity (p.2)

Measurements of cathepsin activity are carried out by analyses of hydrolyzed products from synthetic substrates for cathepsins A, B and C or from proteins for cathepsins D and E.

Cathepsin A activity is determined by micro-titration (Grassman and Heyde[12]) of glutamyl-α- carboxyl liberated from a substrate CBZ-GluTyr. Activities of cathepsins B and C are determined also by microtitration of arginyl carboxyl and phenylalanyl released from the respective synthetic substrates Benzoyl Arg-NH_2 and Gly Phe-NH_2[8] or by quantitation of NH_3 derived from amides released with Conway's micro-diffusion[8, 13]. In the case of cathepsin C, colored peptide derivatives can be used as a substrate[14]. For example, after reaction with GlyPhe-p-nitroanilide, the enzyme activity can be estimated by the quantity of p-nitroaniline released, using a value of 9000 for the molecular extinction coefficient at

410 mμ. Also, cathepsin C activity is determined utilizing a transamidation reaction of this enzyme. This method was proposed by de la Haba et al.[15] and is being widely used. The enzyme is allowed to react with GlyPhe-NH_2 and NH_2OH in the presence of cysteine (β-mercaptoethylamine can be also used); GlyPhe-NH_2 + NH_2OH ---> GlyPhe-NHOH + NH_3. After the reaction the protein is removed with TCA, and $FeCl_3$ solution is added to the supernatant to form a complex salt of ferric hydroxamic acid which is determined by optical absorbance at 540 or 510 mμ.

Hemoglobin or albumin is used as a substrate for the enzyme activity of cathepsin D or E. When albumin is employed, the reaction mixture is deproteinized with TCA and the amount of soluble peptides in the supernatant is determined by the Biuret reaction[9, 11]. In the case of hemoglobin, the supernatant after deproteinization with TCA of the reaction mixture is analyzed by either (1) measurement of optical absorbance at 280 mμ (due to the presence of aromatic amino acids)[8] or (2) determination of tyrosine using the Folin's phenol reagent[16]. Hemoglobin solutions are prepared by the classic method of Anson[16] as follows: red blood cells obtained from cattle blood after removal of both serum and white blood cells are dialyzed against running water to cause hemolysis, and the pH of the hemolyzed solution is adjusted to 3.5 after addition of acetic acid and ammonium sulfate. Urea-denatured hemoglobin is also usable for this purpose[17]. In

this method cattle hemoglobin is denatured with 8M urea in alkaline pH, and the reaction mixture is neutralized with HCl to serve as a substrate solution.

(Note) Glossary for abbreviation used: GlyPhe-p-nitroanilide = glycyl-L-phenylalanine-p-nitroanilide.

IV Individual Cathepsin

(A) Cathepsin A

As has been described previously, cathepsin A hydrolyzes a synthetic substrate, CBZ-GluTyr (carbobenzoxy-L-glutamyl-L-tyrosine)[8]. In this respect, this enzyme displays the same substrate specificity as pepsin. Similar to cathepsin D, cathepsin A is neither activated by cysteine nor inhibited by ICH_2CONH_2 and ICH_2COOH. Because of this similarity, in the fifties when D was not yet identified as a separate enzyme, proteolytic activity (hydrolyzing hemoglobin) of cathepsins was attributed to the action of A[1]. Since cathepsin D was established to be ineffective in hydrolyzing synthetic substrates in the early sixties[10], a number of attempts have been made to characterize the properties of cathepsin A. A highly purified preparation of this enzyme has been recently obtained from cattle spleen by Iodice et al.[18, 19], and a value of 4.85 s (0.9 mg/ml) is given for the sedimentation coefficient of this preparation. The optimum pH for hydrolysis of CBZ-GluTyr is 5.7. Such substrates as CBZ-AlaPhe and CBZ-GluPhe are also hydrolyzed by, but GluTyr is resistant to,

the preparation. Cathepsin A does not hydrolyze proteins on a large scale[19]; which is quite different from cathepsin D. When B-chain of insulin is allowed to react with this enzyme, only one C-terminal amino acid, alanine, is liberated, whereas equal mols of five amino acids (threonine, asparagine, methionine, leucine and tryptophan) are released from the C-terminus of glucagon after reaction with cathepsin A. From these reactions it can be seen that cathepsin A belongs to the class of carboxypeptidases[18]. (p.3)

(B) Cathepsin B

This enzyme hydrolyzes Benzoyl Arg-NH_2[8], and its substrate specificity is the same as that of trypsin. Cathepsin B is a typical SH-enzyme that requires cysteine as an activator. Approximately 200-fold purification of this enzyme from cattle spleen has been recently accomplished[20]. The optimum pH for hydrolysis is about 5.3, and several SH compounds other than cysteine, such as β-mercaptoethylamine, 2,3-dimercaptopropanol, β-mercaptopropanol and glutathione, act as an activator for this enzyme. The activity is inhibited completely by ICH_2COCH_2 (1 mM) but only partially by PCMB (0.1 mM). However, the activity is not affected by such trypsin inhibitors as N-ethylmaleimide and soybean trypsin inhibitor or by dinitrophenol and DFP. In addition to Benzoyl Arg-NH_2, cathepsin B acts on Benzoyl Lys-NH_2 or Benzoyl Arg-OEt but not on Arg-NH_2 or Arg-OEt, indicating that this enzyme requires arginine or lysine, at which amino residue it will

act and that for this purpose arginine or lysine has to be acylated at the α-NH_2 group[1, 20]. Such synthetic peptides as Leu-NH_2, GlyPro, CBZ-GluPhe, GlyGlyGly and CBZ-GlyPhe are apparently not attacked by this enzyme. Cathepsin B is reported to activate trypsinogen to convert to trypsin[21]. Also, cathepsin B is believed to be responsible for the release of chondromucoproteins from rabbit ear cartilage upon autolysis[5, 6]. The enzyme activity in the cartilage is inhibited by arginine or its esters. Cathepsin B found in the pig kidney is capable of hydrolyzing gelatin and β-lactoglobulin at pH 5.0 in the presence of cysteine[22]. Cathepsin B is also found in γ-globulin fraction of sera[23].

This enzyme, similar to cathepsin C, catalyzes not only a hydrolytic but also a transamidation reaction in the following way[24, 25]: Benzoyl Arg-NH_2 + H_2NX ---> Benzoyl Arg-HNX + NH_3 (X = OH or CO of amino acid). The hydrolytic reaction catalyzed by cathepsin B can be in general expressed as follows: Benzoyl Arg-NH_2 + H_2O ---> Benzoyl Arg + NH_3. Thus, the difference between these two reaction systems is whether the acceptor for the transfer of Benzoyl Arg group is H_2O or H_2NX. In other words, it can be said that the hydrolytic and transamidation reaction are essentially the same. However, the optimum pH for the hydrolytic reaction by cathepsin B is 5.3, while that for the transamidation is approximately 6.5[25]. When X = OH; e.g., the acceptor is NH_2OH, a rather high concentration of acceptor (0.4 M) is required for the transamidation reaction. A variety of peptides react with Benzoyl

Arg-NH_2 under the catalytic action of cathepsin B; GlyGly, GlyLeu, Gly-D-Leu, GlyTyr, LeuGly, LeuGlyGly, LeuLeu, GlyGlyGly and GlyLeuLeu. Of these, GlyLeu, GlyTyr and LeuGly are particularly good reactants. However, the transamidation reaction of cathepsin B is much weaker than that of papain or cathepsin C. The lesser degree of transamidation by cathepsin B might be due to the closeness of the optimum pH for hydrolysis and transamidation.

(C) Cathepsin C

Cathepsin C hydrolyzes GlyPhe-NH_2[8], and it was once believed to have the same substrate specificity as chymotrypsin. However, recent studies have indicated that the substrate specificity of cathepsin C is rather limited. This enzyme is activated by cysteine[8, 22, 26], but it is active to some extent in the absence of cysteine. The optimum pH lies between 5.0 and 6.0, and varies with the substrate. The enzyme was at first found in pig kidney[22] but has recently been purified from cattle spleen. In the fifties Tallan et al.[8] and de la Haba et al.[15] succeeded in a partial purification of this enzyme. In the sixties Planta and Gruber[27] carried out further purification of the product obtained by de la Haba's group[15] by the combined use of DEAE-cellulose and DEAE-Sephadex chromatography, but the preparation obtained was still heterogeneous. Recently Metrione et al.[28] have isolated from cattle spleen a product, about 1800 times as potent as the starting material, using gel filtration on (p.4)

Sephadex G-200 and ion exchange cellulose (DEAE and CM) chromatography. The product appears to be homogeneous upon ultracentrifugation with a sedimentation coefficient of 9.73 S, and its molecular weight is estimated to be 210,000 (partial specific volume = 0.73 ml/g). The purified enzyme contains hexoses, hexosamines and sialic acid. It hydrolyzes a number of synthetic substrates which are, according to their susceptibility to the enzyme at pH 6.0, arranged in the following order: GlyTry-NH_2 > GlyTyr-NH_2, GlyPhe-NH_2 > HisPhe-NH_2 > HisTyr-NH_2. Planta and Gruber[29] has studied the nature of the specificity of cathepsin C and shown that the enzyme splits the following part of the substrate molecule:

$$H_2N\overset{R_1}{C}HCONHCH\overset{R_2\downarrow}{C}OX,$$ where R_2 = Phe or Tyr, $\downarrow$ = site of splitting.

It is obvious from the above structure that α-amino group at R_1 position should be free; e.g., R_1 position is the N-terminus. Thus, cathepsin C appears to be quite an unique enzyme which yields dipeptides containing aromatic amino acids after reaction with the substrates. Peptides or proteins which do not possess aromatic amino acids at the penultimate position from the N-terminus would be resistant to this enzyme. The enzyme is found not to hydrolyze hemoglobin, albumin, fibrinogen, oxidized RNase or glucagon, but to release SerTyr from the N-terminus of β-corticotropin. Similar findings have been reported also by Fruton et al.[28]. When a heptapeptide, GlyPhePheTyrThrProLys, is allowed to react with cathepsin C,

the peptide is hydrolyzed to release one after another dipeptides from the N-terminus; GlyPhe then PheTyr[29, 30]. Also, it has been found that the N-terminus of the susceptible substrates to this enzyme is not necessarily to be free α-NH_2 but can be imino (proline) or ϵ-NH_2 (lysine) group[30].

According to Fruton and Mycek[26], cathepsin C is activated by not only cysteine but β-mercaptoethylamine, 2,3-dimercaptopropanol, thioglycholic acid, glutathione and cyanate. However, ascorbic acid is ineffective in this respect. Iodoacetic acid (1 mM) completely inhibits the enzyme activity even in the presence of cysteine, but other common inhibitors such as PCMB, DFP and dinitrophenol are ineffective. The Michaelis constants, K_m, are found to be 10.5 mM for GlyPhe-NH_2, 8.2 mM for GlyTyr-NH_2 and 8.0 mM for AlaTyr-NH_2. Phenylalanine derivatives such as Phe-NH_2 and D-Phe-NH_2 are known to inhibit competitively the activity of this enzyme.

Similar to papain, cathepsin C is reported to cause binding of peptides through its transamidation activity[8, 25, 31, 32], and its activity is more remarkable than that by cathepsin B. The transamidation reaction can be expressed by the following general formula:

$$\text{GlyPhe-NH}_2 + \text{H}_2\text{NX} \longrightarrow \text{GlyPhe-NHX} + \text{NH}_3,$$

where X = OH or CO of amino acid. When molecules of (PheGly)-NH_2 are continually transamidated as a NH_2 donar (H_2NX), polymerization of GlyPhe-NH_2 will occur. Polymerization of GlyPhe units beyond a certain limit causes insolubilization of the

products (polymers)[31, 32]. In addition to GlyPhe-NH_2, GlyTyr-NH_2, AlaPhe-NH_2 and AlaTyr-NH_2 are polymerized by cathepsin C but LeuTyr-NH_2 and GluPhe-NH_2 are not[32]. The polymerization process occurs within the pH range of 6.6 to 7.8, the maximum being observed at pH 7.6[32]. When X = OH; e.g., the NH_2 donar is NH_2OH, hydroxamate is formed as a result of transamidation reaction, and the optimum pH for this reaction is 6.4[31]. The size of the peptides formed by polymerization under the catalysis of cathepsin C varies with the substrates used. For example, polymerization of GlyPhe-NH_2 yields a mixture of octa- and deca-peptides, whereas that of GlyTyr-NH_2 produces in general a decapeptide and AlaPhe-NH_2 forms in general a hexapeptide[32]. During the polymerization process there exists a certain lag period[1], and the formation of oligopeptides as a primer apparently acts as a rate-limiting factor in the process.

Transamidation catalyzed by cathepsin C is, similar to that by B, essentially similar to hydrolysis by the same enzyme and is a transfer of acyl groups. In the hydrolytic reaction the acceptor is H_2O, while in the transamidation reaction H_2O is replaced by H_2NX. It has been confirmed that these two reactions are catalyzed by the same enzyme by Fruton et al.[28] who used a homogeneous cathepsin C preparation.

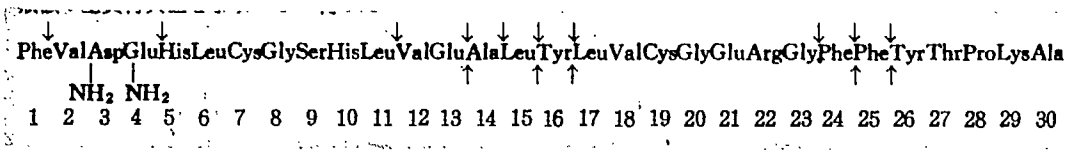
(D) Cathepsins D and E

It has been known for many years that certain intracellular proteinases which have a capacity of hydrolyzing

hemoglobin, do exist in various animal tissues. After Anson[16] had established the method for cathepsin activity using hemoglobin in 1938, the presence of hemoglobin-hydrolyzing activity was reported in a variety of tissues such as cattle liver[33, 34], calf brain[35-37], rabbit striated muscle[38], pig pituitary[39], human red blood cells[40], cattle lung[41, 42] and pig thyroid[43] from 1940 into the fifties. At that time this hydrolytic activity was attributed to the presence in these tissues of cathepsin A which hydrolyzes a synthetic substrate, CBZ-GlyTyr. However, Schäffner and Truelle[34] pointed out that an enzyme preparation obtained from the cattle liver did not split the synthetic substrate, and a possibility that the enzyme responsible for hydrolysis of the synthetic substrate was different from that for hemoglobin hydrolysis, was suggested by Fruton[1]. (p.5)

In 1960 Lapresle and Webb[9] and Press et al.[10] obtained an enzyme preparation from rabbit and cattle spleen, respectively. The enzyme was different from any one of the hitherto known cathepsins A, B and C, did not attack synthetic substrates but only proteins such as hemoglobin and albumin, and was named, therefore, cathepsin D. The optimum pH for hemoglobin hydrolysis is 3.5, and the activity is not affected by ICH_2CONH_2 (1 mM)[10, 44], PCMB (0.1 mM)[10, 44] and EDTA (1.5 mM)[10]. The addition of cysteine (10 mM) does not influence the enzyme activity. None of the following six synthetic peptides is hydrolyzed by the enzyme: Benzoyl Arg-NH_2, GlyTyr-NH_2, CBZ-GluTyr, N-acetyl-DL-phenylalanyl-L-diiodotyrosine,

TyrCys, and CysTyr[10]. Hydrolytic activity of cathepsin D from the cattle spleen toward albumin is 1/4 of that toward hemoglobin, whereas albumin-hydrolyzing activity by the same enzyme from the rabbit spleen is about 5% of the hemoglobin-hydrolyzing activity[44], indicating that the affinity of cathepsin D to albumin is very low. Press et al.[10] have examined the nature of the specificity of this enzyme by the use of B chain of oxidized insulin and concluded that the substrate specificity of cathepsin D is similar to that of pepsin (vide infra);



where ↓ shows the site of splitting by pepsin, while ↑ indicates that by cathepsin D. Dannenberg and Smith[42] have obtained similar hydrolyzed products from B chain of oxidized insulin after reaction with an enzyme preparation obtained from cattle lung. Therefore, the cattle lung preparation is very likely to be cathepsin D.

According to Press et al.[10], cathepsin D contains at least 10 closely related but different compounds. These compounds are separable by CM- or DEAE-cellulose chromatography, and each of these is homogeneous upon electrophoresis or ultracentrifugation. Two of the compounds are reported to have a sedimentation coefficient of 3.3 S and a molecular weight of 58,000. The N-terminal amino acid of the enzyme protein

is identified as glycine. An enzyme preparation with similar multiplicity has been isolated from rat brain[4] and is considered to be cathepsin D. Cathepsin D obtained from the rabbit liver has a molecular weight of 50,000 - 52,000 and its activity is inhibited by 3-phenylpyruvic acid.

Cathepsin E was isolated in 1962 from rabbit bone marrow by Lapresle and Webb[11]. It hydrolyzes human serum albumin with the optimum pH of 2.5 but does not act on such synthetic substrates as CBZ-GluTyr, Benzoyl Arg-NH_2 and GlyPhe-NH_2 in the presence of cysteine (4 mM). Thus, this is a distinctly different enzyme from cathepsin A, B or C. The electrophoretic mobility in agar gel of cathepsin D is estimated to be -1.7×10^{-5} $cm^2V^{-1}sec^{-1}$, whereas that of cathepsin E is -7.2×10^{-5} $cm^2V^{-1}sec^{-1}$. When hydrolytic products of human serum albumin with these two cathepsins are fractionated by gel filtration on a Sephadex G-75 column, the elution patterns are found to be entirely dissimilar. From these findings cathepsin E is confirmed to be an enzyme different from cathepsin D. The activity is hardly affected at pH 2.5, but slightly activated at pH 3.5 - 4.5, by cysteine. Inhibitors such as ICH_2COOH and DFP do not influence the activity.

(E) Other catheptic enzymes (p.6)

Since catheptic enzymes possessing a hemoglobin-hydrolyzing activity were classified under cathepsin D by Press et al.[10], the enzyme obtained by Anson[45] from cattle spleen or

the one described by Dannenberg and Smith[41, 42] has been considered to be identical with cathepsin D.

However, the presence of catheptic enzymes which hydrolyze hemoglobin but which are different from cathepsin D or E, has been reported in various animal tissues. Kalnitsky et al.[46] have isolated a hemoglobin-hydrolyzing enzyme from the pulp cavity of cattle teeth by column chromatography using the following four column matrices: DEAE-cellulose, filter paper impregnated with calcium phosphate gel, calcium phosphate gel - Amberlite and Biogel P-60. The optimum pH for this enzyme is 3.0 - 3.5. The enzyme attacks two peptide linkages between leucine and glutamic acid and between tyrosine and glutamic acid of A chain of oxidized insulin. After reaction with RNase, it releases $\overset{\vee}{a}$ tetrapeptide from the C-terminus. The unique feature of this enzyme appears to split the peptide bond of an amino acid located at the fourth or farther position from the C-terminus of the substrate protein. The enzyme can be fractionated into two units, one having a molecular weight of 41,000 and another 34,000.

Ikezawa et al.[47] have partially purified a hemoglobin-hydrolyzing enzyme from pig liver water extracts using sonic vibration, calcium phosphate gel adsorption, Sephadex G-100 gel filtration and CM-cellulose column chromatography. The optimum pH is approximately 4.4. The enzyme is approximately 70% activated by cysteine (10 mM), but cysteine is not essential for the activity. The activity is inhibited slightly

by ICH_2COOH (1 mM) but completely by o-phenanthroline. Although an about 30% elevation of the enzyme activity is seen in the presence of Fe^{++} (0.7 mM), it is not clear as to whether the ferrous ion is essential for the activity. Reactivity of the enzyme with bovine serum albumin is almost negligible, and no appreciable hydrolysis occurs after 30 min at 37° C. In order to determine whether this enzyme is different from or identical with cathepsin D, further studies are necessary to purify the enzyme to a homogeneous preparation and to examine the nature of the specificity using synthetic substrates.

(Glossary for abbreviations used):

註) 略号 : CBZ-GluPhe : carbobenzoxy-L-glutamyl-L-phenylalanine
CBZ-AlaPhe : carbobenzoxy-L-alanyl-L-phenylalanine
PCMB : *p*-chloromercuribenzoate
Benzoyl Lys-NH_2 : benzoyl-L-lysinamide
Benzoyl Arg-OEt : benzoyl-L-arginine ethyl ester
Arg-NH_2 : L-argininamide
Arg-OEt : L-arginine ethyl ester
Leu-NH_2 : L-leucinamide
CBZ-GlyPhe : carbobenzoxyglycyl-L-phenylalanine
Benzoyl Arg : benzoyl-L-arginine
GlyTry-NH_2 : glycyl-L-tryptophanamide
GlyTyr-NH_2 : glycyl-L-tyrosinamide
HisPhe-NH_2 : L-histidyl-L-phenylalaninamide
HisTyr-NH_2 : L-histidyl-L-tyrosinamide
DFP : diisopropylfluorophosphate
AlaTyr-NH_2 : L-alanyl-L-tyrosinamide
Phe-NH_2 : L-phenylalaninamide
D-Phe-NH_2 : D-phenylalaninamide
AlaPhe-NH_2 : L-alanyl-L-phenylalaninamide
LeuTyr-NH_2 : L-leucyl-L-tyrosinamide
GluPhe-NH_2 : L-glutamyl-L-phenylalaninamide

V Conclusion

Cathepsins are the enzymes that play a certain primary role in post-mortem autolysis of animal tissues. This is due to the fact that the pH value of the post-mortem body medium tends to be in the acidic side (about 4). However, no satisfactory answers are given yet to a question like 'what kind of role(s) would cathepsins play in living tissues ?'. One

theory describes that cathepsins hydrolyze dead cells in the living body to transform into certain materials which can be re-utilized by living cells to synthesize body proteins[1]. However, this can not hold true for at least cathepsin C, since the substrate specificity of this enzyme is so limited that the enzyme would not play any catabolic role. Although both cathepsins B and C are able to polymerize certain peptides through the transamidation reaction, the size of the peptides polymerized is not large enough (up to decapeptides in the case of cathepsin C) to explain satisfactorily the proposed theory. Thus, the physiological significance of (p.7) cathepsins remains to be answered and will be one of the important subjects for future investigation.

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