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Isolation and sequencing of a cDNA clone encoding rat liver lysosomal cathepsin D and the structure of three forms of mature enzymes

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SUMMARY: We isolated and sequenced a cDNA clone corresponding to the entire coding sequence of rat liver lysosomal cathepsin D. The deduced amino acid sequence revealed that cathepsin D consists of 407 amino acid residues (Mr 44,608) and the 20 NH_2-terminal residues seem to constitute a cleavable signal peptide after which 44 amino acid residues follow as a propeptide. Two putative *N*-linked glycosylation sites and aspartic acid in the active site are as well conserved as those of human lysosomal cathepsin D. In the NH_2-terminal sequence analysis of two isolated heavy chains of the mature enzyme, the termini were assigned as tryptophan (118th residue) and glycine (165th or 166th residue), respectively, hence demonstrates that the two heavy chains derive from a split of the single chain of cathepsin D at position between 117th and 118th or between 164th and 165th or 165th and 166th amino acids. We conclude that cathepsin D in rat liver lysosomes is a mixture of three forms composed of a single and two two-chain forms. However, the amounts of the two two-chain forms are low compared with that of the single chain form. Densidometric determination after SDS-PAGE revealed that the two two-chain forms account for less than 5% of the single chain form. There is a 82% similarity in amino acid level between rat and human liver lysosomal cathepsin D.

Cathepsin D, a lysosomal endoprotease present in all mammalian cells (1) belongs to the aspartyl protease family which includes renin, pepsin, and chymosin (2). Cathepsin D is the most abundant protease among the cathepsin group in rat liver lysosomes and functions to degrade intracellular and extracellular proteins, in conjunction with the cooperation with other cathepsin proteases (3, 4).

Huang et al. (5) purified cathepsin D from porcine spleen and revealed that the enzyme was mainly composed of two chain form, heavy (35 kDa) and light chains (15 kDa). Only 5% of the total activity constituted a single chain form (50 kDa). On the other hand, Yamamoto et al. (6) isolated cathepsin D (44 kDa) as a homogeneous form from rat spleen and showed that the cathepsin D contained no two-chain form. We purified cathepsin D from rat liver lysosomes and found that rat liver cathepsin D is a mixture of a single chain and two two-chain forms. In contrast to the case of porcine cathepsin D, the two two-chain forms in rat liver accounted for less than 5 % of the single chain form.

Takahashi and Tang (7) and Shewale and Tang (8) determined the complete structure of the light and heavy chains of porcine spleen cathepsin D, respectively, by the combination of CNBr cleaved peptides

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Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; CBB, Coomassie brilliant blue.

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and Edman degradation. Faust et al. (9) cloned and sequenced a cDNA for human cathepsin D using as a probe a human renin exon 3 genomic fragment (10, 11). In this paper, we attempted to clarify the relationships among the three forms of rat liver cathepsin D using the cDNA sequencing and amino acid sequencing determined by Edman degradation. Also, we compared structural relationships among the three cathepsin D isolated from three species.

MATERIALS AND METHODS

Purification of cathepsin D from rat liver lysosomal contents and preparation of antibody against cathepsin D

Rat liver lysosomal contents were prepared according to the method of Ohsumi et al. (12). Cathepsin D in the lysosomal contents was concentrated by adding ammonium sulfate to make a 65% saturation. The resulting pellets obtained by centrifugation were dissolved in 50 mM sodium acetate buffer, pH 5.0, and the solution was dialyzed against the same buffer. The dialysate was applied to pepstatin-Sepharose (13) and cathepsin D was purified to homogeneity, essentially as described by Afting and Becker (14). As shown in Fig. 1, the purified cathepsin D was separated into four bands on SDS-PAGE. The 43 kDa band stained with CBB on SDS-PAGE was excised and homogenized in 20 mM Na_2CO_3 containing 0.15 M NaCl. The homogenate was centrifuged at 10,000xg for 10 min to obtain pellets and the pellets were again homogenized in the same solution. The pellets obtained by recentrifugation were mixed with the same solution containing 0.2 % SDS. The suspension was centrifuged for 60 min at 105,000xg and the resulting blue supernatant containing 43 kDa protein was precipitated by adding TCA. The TCA precipitate obtained by brief centrifugation (10,000 rpm, 5 min) was dissolved in 20 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl. This solution was used to raise antibody against cathepsin D in rabbits.

Protein sequence analysis

The NH_2-terminal sequences of proteins were determined in an Applied Biosystem 470A protein sequencer/Spectra Physics SP8100 HPLC system. The purified cathepsin D was separated into 43 kDa, 34 kDa, 30 KDa, and 12 kDa peptides on SDS-PAGE. Western blot analysis using antibody against the 43 kDa cathepsin D revealed that the other two bands (34 kDa and 30 kDa) were also immunoreactive (data not shown), hence these two bands could be part of the 43 kDa cathepsin D. However, the smallest band (12

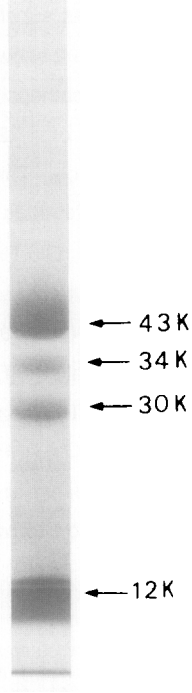

Fig. 1. Polyacrylamide gel electrophoresis of the purified rat liver lysosomal cathepsin D in the presence of SDS. Gel was stained for proteins with Coomassie blue.

kDa) is not reactive to the antibody. Probably, the peptide is too small to be retained on nitrocellulose compared to the other three peptides during blotting from the gel. Each band after SDS-PAGE was cut out and extracted from the polyacrylamide gel and each extract in 20 mM Na_2CO_3 containing 0.15 M NaCl and 0.2 % SDS was precipitated by adding TCA to make a 10 % TCA solution. The pellet was washed several times with acetone and dried at room temperature. The resulting pellet (about 10 μg protein) was dissolved in 30 ml of 0.3 % SDS and subjected to NH2-terminal sequencing in an Applied Biosystem 470 A protein sequencer/Spectra Physics SP8100 HPLC system.

Screening of the cDNA library

A rat liver cDNA expression library (15) in λgt11 was screened with specific rabbit antibodies raised against the 43 kDa cathepsin D, using horseradish peroxidase-conjugated anti-rabbit IgG as the second antibody, as described elsewhere (16). From the $1.2x10^5$ plaques screened, 4 putative positive clones were obtained. The one with the longest insert (1.9 kbp) was used for DNA sequencing because the open reading frame of human cathepsin D is approximately 1.2 kbp. The clone with 1.9 kbp insert (cat-1) was subcloned into the plasmid vector pUC 118 and analyzed by restriction mapping.

DNA sequencing

Restriction endonuclease fragments of cDNA of cat-1 were sub-cloned into plasmid vector pUC118. Nucleotide sequences were analyzed by the dideoxynucleotide chain termination method (17), in which denatured plasmid DNA was used as a template as described (18).

Computer analysis of cDNA and protein

Nucleotide and protein sequences were analyzed using the GENAS System at Kyushu University Computer Center (19).

RESULTS AND DISCUSSION

Isolation of cDNA clones

From a rat liver cDNA library constructed with λgt11 as vector (15) and screened with anti-cathepsin D antibodies we isolated four positive clones from approximately $1.2x10^5$ phages. The clone containing the longest insert was termed cat-1 (insert length, 1,879 bp).

Sequence analyses of the cDNA and structure of cathepsin D

The longest cDNA fragment (cat-1, 1,879 bp) was subcloned into the plasmid vector pUC118 and analyzed by restriction mapping (data not shown). Fig. 2 shows the nucleotide sequence determined from the cat-1-cDNA and the deduced primary structure of cathepsin D. As shown in Fig. 2, composition of the cat-1-cDNA sequence has an open reading frame of 1,224 bp (nucleotides 8-1,232) flanked by 8 nucleotides of 5'- and 627 of 3'-untranslated sequence. This clone has no poly(A) tail. However, the presumed polyadenylation signal is located on nucleotides from 1,863 to 1,868. The NH_2-terminal sequence determined from the 43 kDa single form, Glu-Pro-Val-Ser-Glu-Leu-Leu-Lys-Asn-Tyr-, was identical to the amino acid sequence starting at 65th glutamine. As shown in Fig. 1, there are two two-chain forms in rat liver lysosomal cathepsin D, the larger one (34 kDa) gives the NH_2-terminal sequence, Trp-Val-His-His-Lys-Tyr-Asn-Ser-Asp-Lys-, by Edman degradation. This sequence corresponds to the deduced sequence which started from the 118th tryptophan. The heavy chain in the other two-chain form gives NH_2-terminal sequences of Gly-Gly-Ile-Lys-Val-Glu-Lys-Gln-Ile-Phe- and Gly-Ile-Lys-Val-Glu-Lys-Gln-Ile-Phe- which correspond to the deduced sequence starting either at the 165th or 166th glycine. We tentatively conclude that a single form cathepsin D is converted to two two-chain forms. The heavy chain with larger

Fig. 2. The nucleotide sequence and deduced amino acid sequence of rat cathepsin D cDNA. The amino acid sequence obtained from protein sequencing is boxed. Putative signal sequence and propeptide are shown broken underline and underline, respectively. (▲) and (◆) indicate the proteolytic cleavage sites. Potential N-glycosylation sites, active aspartyl groups, the termination codon and polyadenylation signal are indicated by #, ✱, ---, and ✱✱✱, respectively.

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1     CC GCG ACC ATG CAG ACC CCC GGC GTC TTG CTG CTC ATT CTC GGC CTC
                 Met Gln Thr Pro Gly Val Leu Leu Leu Ile Leu Gly Leu

48    CTG GCT GCG TCC TCC TCC GCG CTT ATC AGA ATC CCC CTG CGC AAG TTC
14    Leu Ala Ala Ser Ser Ser Ala Leu Ile Arg Ile Pro Leu Arg Lys Phe

96    ACA TCC ATC CGT CGG ACT ATG ACG GAA GTG GGA GGC TCT GTG GAA GAC
30    Thr Ser Ile Arg Arg Thr Met Thr Glu Val Gly Gly Ser Val Glu Asp

144   CTG ATC CTT AAA GGT CCC ATA ACC AAG TAC TCC ATG CAG TCA TCT CCT
46    Leu Ile Leu Lys Gly Pro Ile Thr Lys Tyr Ser Met Gln Ser Ser Pro

192   AGG ACC AAG GAG CCA GTG TCA GAG TTA CTA AAA AAC TAC CTG GAT GCC
62    Arg Thr Lys Glu Pro Val Ser Glu Leu Leu Lys Asn Tyr Leu Asp Ala

240   CAG TAC TAT GGT GAG ATC GGC ATT GGG ACT CCC CCA CAG TGT TTC ACA
78    Gln Tyr Tyr Gly Glu Ile Gly Ile Gly Thr Pro Pro Gln Cys Phe Thr

288   GTC GTC TTT GAC ACT GGC TCC TCT AAC CTG TGG GTC CCC TCC ATT CAT
94    Val Val Phe Asp Thr Gly Ser Ser Asn Leu Trp Val Pro Ser Ile His

336   TGC AAG CTG CTG GAC ATA GCC TGC TGG GTC CAC CAC AAG TAC AAC AGT
110   Cys Lys Leu Leu Asp Ile Ala Cys Trp Val His His Lys Tyr Asn Ser

384   GAC AAG TCC AGC ACC TAT GTG AAG AAT GGC ACA TCC TTC GAC ATC CAC
126   Asp Lys Ser Ser Thr Tyr Val Lys Asn Gly Thr Ser Phe Asp Ile His

432   TAC GGC TCA GGT AGC CTC TCT GGG TAC CTG AGC CAG GAC ACT GTG TCG
142   Tyr Gly Ser Gly Ser Leu Ser Gly Tyr Leu Ser Gln Asp Thr Val Ser

480   GTT CCA TGT AAG TCA GAC TTA GGA GGT ATC AAG GTG GAG AAA CAG ATC
158   Val Pro Cys Lys Ser Asp Leu Gly Gly Ile Lys Val Glu Lys Gln Ile

528   TTT GGG GAA GCC ACC AAG CAG CCT GGA GTC GTA TTC ATC GCA GCC AAG
174   Phe Gly Glu Ala Thr Lys Gln Pro Gly Val Val Phe Ile Ala Ala Lys

576   TTT GAT GGC ATC TTG GGC ATG GGC TAC CCT TTT ATC TCT GTT AAC AAT
190   Phe Asp Gly Ile Leu Gly Met Gly Tyr Pro Phe Ile Ser Val Asn Asn

624   GTG CTC CCG GTC TTC GAC AAC CTG ATG AAA CAG AAG CTG GTG GAA AAG
206   Val Leu Pro Val Phe Asp Asn Leu Met Lys Gln Lys Leu Val Glu Lys

672   AAC ATC TTC TCC TTC TAC CTG AAC AGG GAC CCA ACC GGG CAA CCT GGA
222   Asn Ile Phe Ser Phe Tyr Leu Asn Arg Asp Pro Thr Gly Gln Pro Gly

720   GGA GAA CTA ATG CTT GGC GGC ACT GAC TCC AGA TAC TAC CAC GGG GAG
238   Gly Glu Leu Met Leu Gly Gly Thr Asp Ser Arg Tyr Tyr His Gly Glu

768   CTG TCC TAC CTG AAC GTC ACC CGA AAC GCG TAC TGG CAG GTG CAC ATG
254   Leu Ser Tyr Leu Asn Val Thr Arg Asn Ala Tyr Trp Gln Val His Met

816   GAC CAG CTG GAG GTG GGC AGC GAG CTG ACT CTG TGC AAG GGA GGC TGT
270   Asp Gln Leu Glu Val Gly Ser Glu Leu Thr Leu Cys Lys Gly Gly Cys

864   GAG GCT ATT GTG GAC ACA GGG ACG TCT CTT CTG GTG GGG CCT GTG GAC
286   Glu Ala Ile Val Asp Thr Gly Thr Ser Leu Leu Val Gly Pro Val Asp

912   GAG GTG AAG GAA CTA CAG AAG GCC ATT GGG GCA GTG CCT CTC ATC CAG
302   Glu Val Lys Glu Leu Gln Lys Ala Ile Gly Ala Val Pro Leu Ile Gln

960   GGC GAG TAT ATG ATC CCT TGT GAG AAG GTG TCC AGC CTG CCC ATT ATC
318   Gly Glu Tyr Met Ilu Pro Cys Glu Lys Val Ser Ser Leu Pro Ile Ile

1008  ACC TTT AAG CTA GGA GGC CAA AAC TAT GAA CTA CAC CCA GAG AAG TAC
334   Thr Phe Lys Leu Gly Gly Gln Asn Tyr Glu Leu HIs Pro Glu Lys Tyr

1056  ATA CTC AAG GTA TCG CAG GCT GGA AAG ACG ATC TGC CTG AGT GGC TTC
350   Ile Leu Lys Val Ser Gln Ala Gly Lys Thr Ile Cys Leu Ser Gly Phe

1104  ATG GGG ATG GAC ATA CCC CCT CCC AGT GGG CCG CTC TGG ATC CTG GGC
366   Met Gly Met Asp Ile Pro Pro Pro Ser Gly Pro Leu Trp Ile Leu Gly

1152  GAT GTC TTT ATT GGC TGC TAC TAC ACC GTG TTT GAC AGA GAA TAC AAT
382   Asp Val Phe Ile Gly Cys Tyr Tyr Thr Val Phe Asp Arg Glu Tyr Asn

1200  AGG GTC GGC TTT GCC AAG GCT GCC ACA CTC TAA CTTGCTCCTTCTTCACTGT
398   Arg Val Gly Phe Ala Lys Ala Ala Thr Leu ---

1252  CAGGGAACTGGATCAGAGTCCAGTAGAGAAAGCCAGCCAGCCCCATCCCTCCACCTGCCCCAC
1315  TCACACATATTCACACTCGCCTAGTGTTGCTGGCCCTTGGGAGGCCTGGCTGGACTGGTCCAG
1378  CTGTTCTGTTCTGTGGTCCCTGTCCCTGGGTTCAGATTGCTGCCCTCCGCCTGTCTGAAGGAG
1441  GCCAAGGCCTACCCAGTACAAAAGGCTGCCTTTAAAGGCCCCTACTGGCTTGGTAGCTGCCGG
1504  GATGGATTGTCTTGTCCTGCTGCCCTTTGCTGCGTGGGCAGCACTCTGAAGCAGGCAAATGGG
1567  TCTTGGGATCCCTCCCAGAAACCTGCTCTGACCAGACCCTCAGGCAGCCTGGGGATGGCACCA
1630  AGCTCTACTGCCCCACTATCTCTGGCCAGGCAAAGGCTGAAGGTGAGCAGAAGGAGCAAGAGG
1693  ACAGAACGGAAAACTATATGAACCTGGGGGGGGTTACCTGGGACCTGACCCCGACCTCCTGGG
1756  AAGGCATGCTTCAGCCTGGGGCAGAGGTAGGATGGCTGACTGGTTTTGGCTGGCCTCTGCTGC
1819  CCTCATCCTGGGCTAGTCAGATGGGAGCCAAAGTTGATATACAAATAAAGTTGTTTTGGG
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molecular weight was the results of a proteolytic cleavage by lysosomal endoprotease between the 117th cysteine and the 118th tryptophan of the single chain form. The other heavy chain with a smaller molecular weight is also produced by proteolytic hydrolysis either between the 164th leucine and the 165th glycine or the 165th glycine and the 166th glycine. Since the NH_2-terminal sequence of the smallest band with 12 kDa is the same as that of 43 kDa peptide, we concluded that the 12 kDa peptide is a mixture of light chains. Biosynthetic studies of cathepsin D have shown it to be synthesized with pre- and propeptide regions (20, 21). The NH_2-terminal region presented here consists of numerous hydrophobic amino acids characteristic of a signal peptide. Erickson et al. (20) found that the prepiece of porcine cathepsin D is 20 amino acid long. Faust et al.(9) proposed that the prepiece of human cathepsin D is 20 amino acids long based on the works done by Erickson et al. (20) and Gieselman et al. (21). We assumed the signal sequence of rat cathepsin D to be from the first methionine to the 20th alanine and the area from leucine to the 64th lysine we conclude to be propeptide because the single chain form begins after the lysine. It was evident that the cDNA for rat liver cathepsin D is coding for 407 amino acids (44,608 Da), of which 342 (37,682 Da) residues constitute the entire length of the mature protein and 60 (6,926 Da) a NH_2-terminal peptide which is split into 20 (1,937 Da) of a signal peptide and 45 (4,988 Da) of propeptide, respectively. The predicted

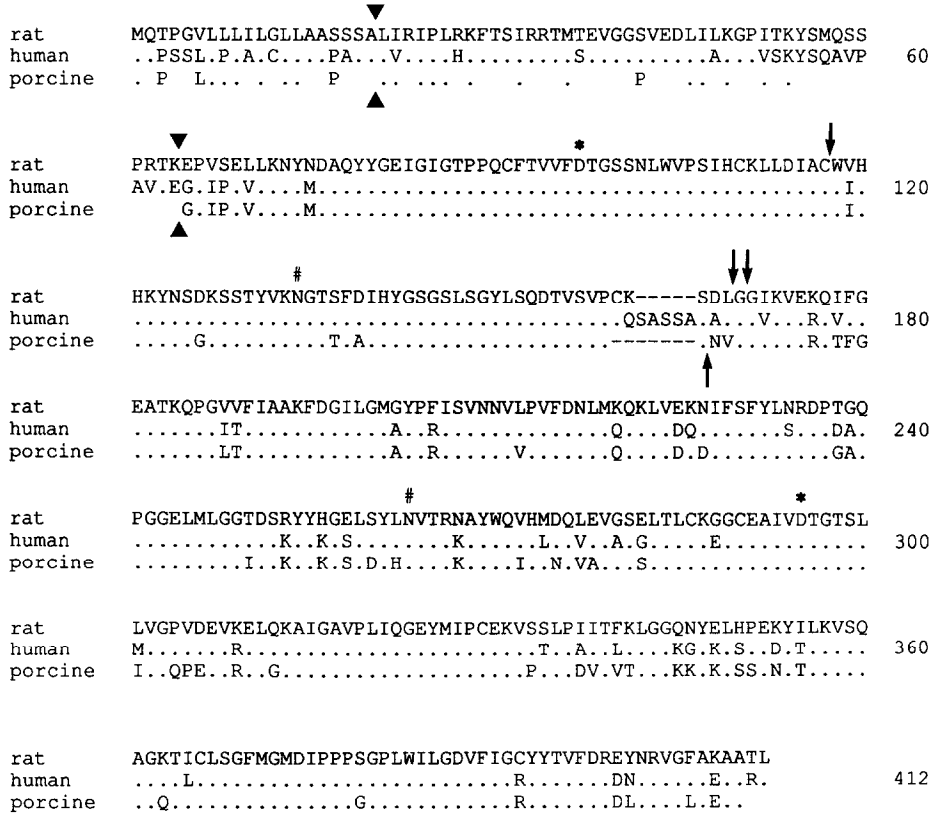

Fig. 3. Alignment of amino acid sequences of rat, human, and porcine cathepsin D. Dots in the lower two sequences indicate identity with the rat cathepsin D sequence. Gaps, shown by dashes, were introduced for optimal alignment. Porcine cathepsin D was separated into the light and heavy chains at the dashes but rat cathepsin D not at the dashes. The dashes in rat cathepsin D also show a deletion portion of the enzyme where the human cathepsin D is filled with amino acid residues. Blanks in the porcine sequence indicate amino acids not determined. Arrowheads denote the cleavage sites of signal sequence and propeptide. Arrows indicate the splitting sites of rat and porcine cathepsin D. Potential *N*-glycosylation sites and active aspartyl groups are indicated by (#) and (*), respectively.

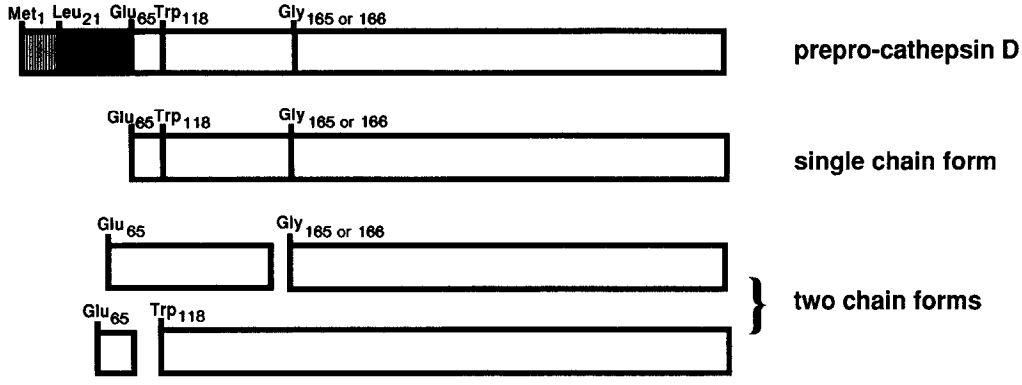

Fig. 4. Schematic representation of proteolytic processing of rat cathepsin D. Vertical straight lined area shows a signal peptide and hatched area the propeptide.

sequence contained 2 potential *N*-glycosylation sites at positions 134 and 258 and the active site aspartyl residues are also well conserved, as is the case with other aspartyl proteases.

Comparison of rat, human, and porcine cathepsin D

Porcine spleen cathepsin D and rat liver cathepsin B, H, L, are mostly composed of heavy and light chains (5, 22-24), the so-called two-chain form. However, cathepsin D purified from the rat spleen contains only a single chain form (6) and as can be seen in Fig. 1, cathepsin D purified from rat liver contains an abundant single chain form and small amounts of two two-chain forms. One two-chain form was composed of a 34 kDa heavy chain and a 9 kDa light chain while the other one has 30 kDa and 14 kDa heavy and light chains, respectively. The deduced amino acid sequence of the mature cathepsin D has an 82% homology to those of human and porcine cathepsin D. The propeptides from rat and human cathepsin D have a 72% homology. Despite such a high homology, rat cathepsin D polypeptide is shorter than that in human's by 5 amino acid residues. Five amino acids from 162 to 166 residues of the human enzyme are deleted in the rat one, and the deletion is near the splitting position which generates the two-chain form in the rat enzyme (Fig. 3). Faust et al.(9) found that porcine cathepsin D is 7 amino acid residues shorter than that in human's and suggested that these 7 residues are lost during proteolytic conversion from the single to the two-chain form. In this case, 7 amino acids from 160 to 166 residues of the human enzyme lacked in the porcine one (Fig. 3). Since the splitting sites which generate the two-chain forms in rat and porcine cathepsin D, respectively, are located in close positions in each sequence, the lack of 7 amino acid residues of the porcine enzyme may be a result of deletion in a genomic level, as in the case of rat one. Since Faust et al. (9) suggested that the similarity in amino acid sequences between cathepsin D and renin, pepsin, and chymosin was 46,49 and 47%, respectively, we examined the similarity between the whole sequence of rat cathepsin D and human cathepsin E (25). We revealed that there is a 46% similarity and aspartic acid in active sites and 6 cysteines are well conserved, hence the three-dimensional structure in this family of aspartyl proteases is conserved. The proteolytic split sites of rat cathepsin D are shown in Fig. 4.

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