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The Selectivity of Cathepsin D Suggests an Involvement of the Enzyme in the Generation of T-cell Epitopes*

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The selectivity of cathepsin D, a mammalian intracellular aspartyl proteinase involved in the degradation of endocytosed proteins, was studied. For this purpose, several proteins of known primary structure were subjected to mild proteolysis by the enzyme, and the preferentially cleaved peptide bonds were identified. Comparison of the primary structures around these sites indicates that cathepsin D shows a strong preference for peptide bonds within a distinct sequence pattern of amino acids extending over 7 residues. In general, this pattern is most likely to occur within amphipathic α-helical structures.

These findings and their possible implications are discussed together with additional evidence suggesting an important role for cathepsin D in the processing of protein antigens, an essential step for their recognition by T-cells. Accordingly, it is proposed that the proteolytic activity of cathepsin D is crucial in selecting processing sites and hence the location and structural context of T-cell epitopes for the majority of protein antigens.

Cathepsin D (EC 3.4.23.5) is an aspartyl proteinase found in virtually all mammalian tissues (for a review, see 1 and 2). The enzyme is one of the more dominant proteases within both lysosomal and endosomal vesicles (3). So far, evidence has been reported that cathepsin D plays a role in the breakdown of connective tissues (2) and is responsible for the conversion of procollagen to collagen (4), the cleavage of β-endorphin and β-lipotropin (5), parathyroid hormone degradation (6), and the inactivation of cystatins (7). Apart from these specific functions, cathepsin D has been found to fulfill a more general role in the proteolytic degradation of various endocytosed proteins, *e.g.* of hemoglobin (8), serum albumin (9), and myoglobin.[1] This role of cathepsin D may be of immunological significance.

Processing of protein antigens, which involves limited proteolysis, is generally required for their recognition by T-cells (for a recent review, see 10). This processing takes place within antigen-presenting cells that subsequently display the resulting fragments on their surface. In most cases, T-cell recognition of a protein is focused on a limited number of sites, the so-called T-cell epitopes, which form part of these fragments. Information on the proteases that are involved in antigen processing and on their substrate specificity will be of help in predicting the products of processing and, hence, the location and structural context of T-cell epitopes. It is expected that this will facilitate the development of synthetic immunogens and vaccines.

Reports on the substrate specificity of cathepsin D from various sources indicate that the enzyme is optimally active at pH 3–4 and that it preferably cleaves peptide bonds linking two hydrophobic amino acid residues (2, 11). In this respect, no major differences were found among preparations of the enzyme from different species nor from different tissues within one species (1, 2, 12, 13). Like other aspartyl proteinases, cathepsin D appears to recognize its substrate over a relatively large area, extending over possibly as many as 7 amino acid residues (1). Peptide substrates of less than 5 amino acid residues are not cleaved by the enzyme (11, 14). This suggests that the specificity of the enzyme depends not only on the amino acid residues directly flanking the scissle bond but also on primary or even higher order structural features of the substrate which involve other amino acids. Rat liver cathepsin D has been reported to show a preference for peptide bonds within regions with a low potency for β-turn formation (13). However, this study, like many others (reviewed in 1), involved proteolysis at pH 3.5 during prolonged incubation at 37 °C. As the stability of secondary or higher order protein structures under such acidic conditions may be questionable, a possible effect of certain structural features upon recognition by cathepsin D may not have been fully revealed by these studies. Moreover, allowing a protein substrate to be cleaved nearly to completion renders the interpretation of cleavage data rather difficult. For instance, after extensive proteolysis, one cannot distinguish between cleavage sites initially recognized as part of an intact protein molecule and those recognized only after exposure as a result of preceding cleavages. This distinction may be relevant as the acidic conditions under which cathepsin D functions *in vivo*, especially within endosomes, are relatively mild (15). In the latter case, an environment of pH 5–6 limits the activity of cathepsin D, and consequently proteins that are processed within endosomes presumably will suffer cleavage only to a limited extent. Furthermore, many proteins will retain their native structure at this pH. Very few reports have appeared on the selectivity of cathepsin D under correspondingly mild conditions (*e.g.* 16).

In this paper we report on a study of the selectivity of bovine spleen cathepsin D during limited proteolysis of various substrates of known primary structure. Proteins used in this study are sperm whale myoglobin, hen egg-white lysozyme, ovalbumin, bovine serum albumin, bovine cytochrome *c*, pigeon cytochrome *c*, and elongation factor Tu from *Escherichia coli*. Although the two cytochrome *c* variants share highly homologous sequences, they are considered as two distinct protein substrates in this study. Digestions were

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performed under mildly acidic conditions with a large excess of protein substrate, and they were kept short to limit the number of cleavages per protein molecule. Under these conditions, the majority of cleavages will reflect the activity of the enzyme upon the intact protein molecule. A possible preference of cathepsin D for extended primary and/or stable secondary structural features within protein substrates may thus be revealed. With the above protein substrates, 17 preferentially cleaved peptide bonds were identified. Comparison of the protein structures involved suggests a preference of bovine cathepsin D for peptide bonds within a distinct structural context not noted previously.

MATERIALS AND METHODS

Purified bovine spleen cathepsin D (12.8 Anson units/mg), ovalbumin, bovine cytochrome *c*, and pigeon cytochrome *c* were purchased from Sigma; sperm whale myoglobin from Serva; and hen egg-white lysozyme and bovine serum albumin from Boehringer Mannheim. Sperm whale myoglobin was further purified according to Hapner *et al.* (17); all other proteins were used without further purifications. Highly purified elongation factor Tu from *E. coli* was the kind gift of Dr. B. Kraal (State University of Leiden, The Netherlands). Acetonitrile was obtained from J. T. Baker Chemical Co. and trifluoroacetic acid from Pierce Chemical Co. The other chemicals were of analytical grade.

Digestions were performed by adding 40 μl of a cathepsin D stock solution (1 mg/ml) to 1 ml of buffered substrate solution containing 2 mg of protein and incubating this mixture at 37 °C for a suitable period of time (see "Results"). The buffer used throughout was 80 mM sodium acetate adjusted with acetic acid to the desired pH (see "Results"). After digestion, cathepsin D was inactivated by adding Tris/HCl buffer pH 9 to a final concentration of 300 mM and acetonitrile to a final concentration of 50% (v/v). The addition of acetonitrile resulted in the precipitation of uncleaved protein and very large protein fragments (over about 50 residues) that would obstruct analysis of the digest on the standard octadecyl reversed-phase columns. The mixture was kept overnight at room temperature, and the precipitate was removed by centrifugation immediately prior to HPLC[2] analysis.

Analysis of the digests and the purification of peptides were achieved by reversed-phase HPLC on a homemade Hypersil ODS column (5-μm particles, 5 × 250 mm). For all separations, acetonitrile gradients in trifluoroacetic acid (0.1%, v/v) were used. Amino acid analysis of peptides was performed by HPLC analysis of phenylthiocarbamyl-amino acids as described by Janssen *et al.* (18). Sequencing of peptides was performed on an Applied Biosystems model 470A protein sequenator equipped with a model 120A PTH Analyzer.

RESULTS

Mild Digestion with Cathepsin D—For most of the protein substrates studied, incubation with cathepsin D under mildly acidic conditions led to the rapid release of fragments, among which only a few were obtained in relatively high yield after an incubation period of 1 or 2 h. The HPLC elution profiles of the various digests are given in Fig. 1. Because individual proteins differ significantly in their overall susceptibility to cleavage by cathepsin D (19), the pH and incubation time required to achieve a limited but detectable extent of cleavage were different for each protein (see legend to Fig. 1). Identification of the preferentially released protein fragments was achieved by amino acid analysis and, in some cases, by sequence determination of the NH_2-terminal 4 amino acid residues. The results obtained are summarized in Table I.

One of the protein substrates used, hen egg-white lysozyme, was found to be extremely resistant to cleavage by bovine cathepsin D, and in fact no significant cleavage could be detected over a 24-h period at pH 4 (results not shown). In the case of ovalbumin, cleavage by cathepsin D at pH 5 did occur, but no dominant cleavage product(s) could be discerned; no peptides were detected in the fractions containing major *peaks 1–3* (see Fig. 1*A*).

In other cases too, a significant absorption peak in the HPLC elution profile was caused by nonpeptide material of unknown origin or by mixtures of co-eluted peptides, each in relatively low yield (relative molar yield less than 2%). In one case (bovine serum albumin, *peak 3*), the co-eluted peptides could not be separated by additional purification steps, and the mixture as such was subjected to sequencing analysis. The result revealed the presence of at least three and possibly as many as five different peptides in the mixture represented by this peak, each in low yield (data not shown). No further attempts were undertaken to identify the low yield peptides found within the different mixtures.

Preferential Cleavage Sites—A cleavage site was considered preferential when the relative molar yield of one or both of the cleavage product(s) flanking that site exceeded the arbitrary threshold of 4%. Comparison of the 17 preferential cleavage sites at the level of primary structure (Table II) reveals the presence of similar residues at distinct positions near the cleaved peptide bond. These residues are enclosed by boxes in Table II. Nearly all preferentially cleaved peptide bonds (16/17) were found to link hydrophobic residues. The aromatic residues tryptophan, tyrosine, and phenylalanine together with leucine dominate the P_1 position (15/17). The other hydrophobic amino acids were found much less frequently at this position, suggesting that a relatively small amino acid side chain is less favored by cathepsin D at the P_1 position. In addition, hydrophobic residues are clearly preferred at the P_1 site (16/17). However, the structural requirements for this position seem to be less strict, as is illustrated by the larger variety of residues present at this site. A preference for a hydrophobic residue similarly dominates the P_2 position (13/17), albeit less strongly. A highly polar residue such as glutamic acid apparently can also be accommodated at this position. These features are very much like those reported for the specificity of rat liver cathepsin D (13).

In addition, the results as listed in Table II strongly suggest that bovine cathepsin D also recognizes residues at the P_2' and P_5' positions. At both positions, a highly polar or charged residue appears to be preferred by the enzyme (15/17 and 11/17, respectively). In most cases, a basic residue is involved (11/17 and 10/17, respectively). The structural requirement at the P_2' and P_5' positions is not absolute, and other polar or small residues (threonine, alanine, or glycine) may occupy these positions. However, all major cleavage sites identified thus far contained at least 1 basic residue at either the P_2' or the P_5' position.

DISCUSSION

The data reported here include the identification of peptide bonds within different proteins that are highly susceptible to cleavage by bovine spleen cathepsin D under mild conditions. Obviously, the susceptibility of a particular peptide bond to mild proteolytic cleavage will depend not only on the substrate specificity of the enzyme used but also on the accessibility of the peptide bond in the protein structure. The contribution of the substrate specificity *per se*, however, may be highlighted by comparing preferential cleavage sites in a number of different protein substrates. In the present study, such a comparison clearly revealed a preference of cathepsin D for peptide bonds within a distinct structural context. The two hydrophobic amino acid residues flanking the cleaved peptide bond are generally preceded by a third hydrophobic residue

[2] The abbreviation used is: HPLC, high pressure liquid chromatography.

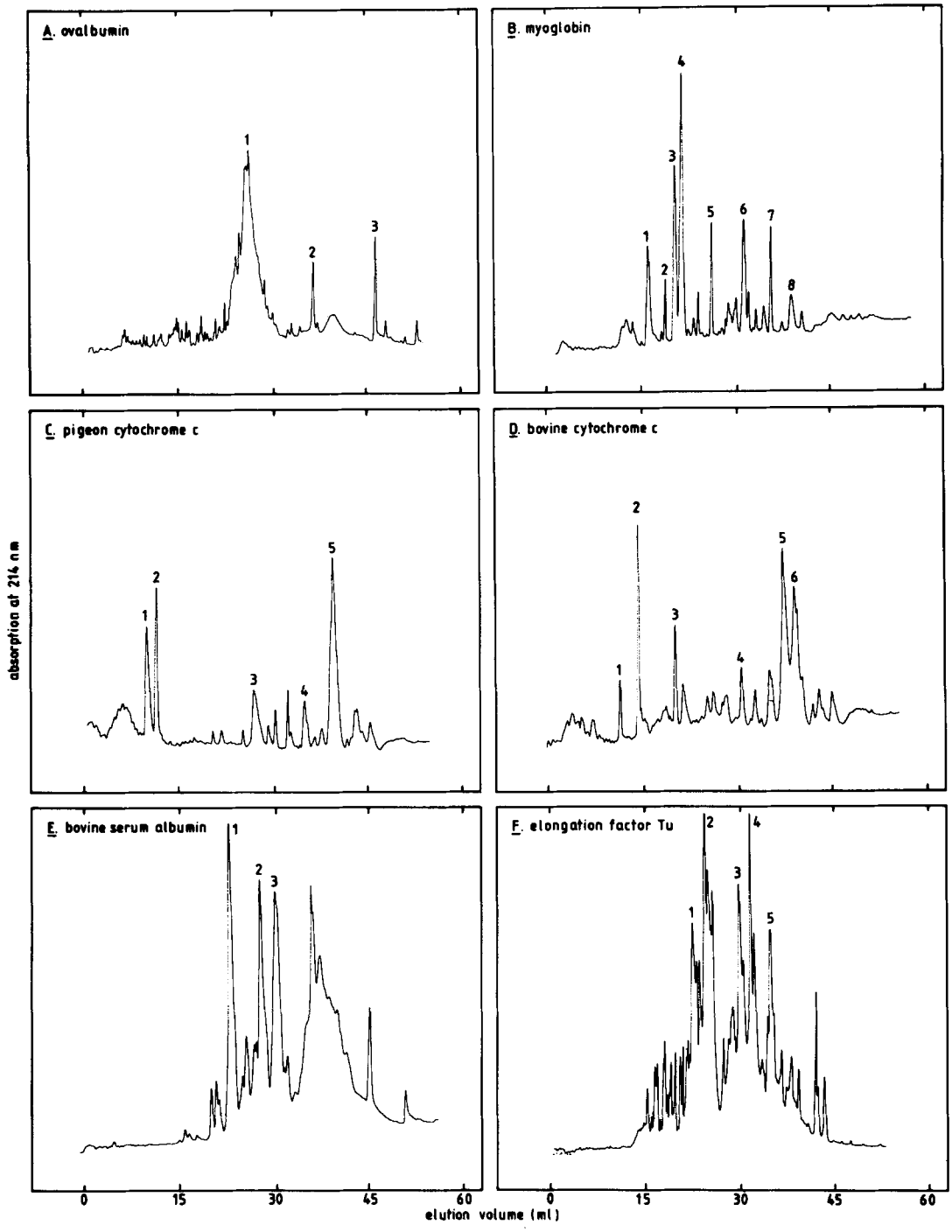

FIG. 1. **Reversed-phase HPLC profiles of various protein digests obtained after mild treatment with bovine spleen cathepsin D at 37 °C.** The following conditions were used: for ovalbumin, 1 h, pH 5.0; for myoglobin, 1 h, pH 5.0; for pigeon and bovine cytochrome *c*, 2 h, pH 4.0; for bovine serum albumin, 2 h, pH 5.0; and for elongation factor Tu, 1 h, pH 4.0. Protein fragments were separated on a Hypersil ODS reversed-phase column by the application of a linear gradient of acetonitrile (10–70% (v/v) over 90 min) in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1.07 ml/min. For further details, see "Materials and Methods."

at the P_2 position and followed by a charged residue in the P_2' position. Another charged residue is frequently found at the P_5' position; at both positions, the charged residue very often is a basic one. This common structural motif around the cleavage sites is depicted in Fig. 2.

It should be noted that in the present study preferential cleavage sites are identified through an analysis of digestion products that are rapidly released from a protein substrate. Obviously, these sites are not the only peptide bonds that are susceptible to cleavage by cathepsin D since other peptides are also released under the present conditions, albeit in substantially smaller amounts. In addition, a very large number of peptides appears upon prolonged incubation of all the protein substrates used here except lysozyme. Most of the initially dominant cleavage products are also subject to slow continued degradation. Apparently, their dominant presence during the early stage of digestion is transient and reflects the selectivity of cathepsin D.

The resistance of hen egg-white lysozyme to cleavage by cathepsin D is in line with the requirement for an accessible

TABLE I

Identification of the various protein fragments preferentially released by bovine spleen cathepsin D

Peak designation refers to Fig. 1. In all cases, identification was achieved by amino acid analysis. For some peptides (marked *), the first 4 amino acids were also identified by sequencing.

Protein substrate	Peak No.	Residues	Relative molar yield
			%
Myoglobin	1	12–14	3.1
	2	8–11	1.6
	3*	15–29	10.6
	4*	138–153	11.2
	5*	2–7	1.5
	6*	112–137	8.6
	7	2–11	1.1
	8*	70–100	3.3
Pigeon cytochrome *c*	1	98–104	6.1
	2	Nonpeptide matter	
	3	83–97	6.8
	4	68–82	4.5
	5	83–104	13.8
Bovine cytochrome *c*	1	98–104	6.1
	2	Nonpeptide matter	
	3	95–104	1.2
	4	83–97	2.6
	5	68–82	8.2
	6	83–104	7.3
Bovine serum albumin	1	431–445	18.3
	2	354–368	9.0
	3*	Mixture of peptides	
Elongation factor Tu	1	Mixture of peptides	
	2	1–35	12.4
	3	Mixture of peptides	
	4	312–331	14.0
	5	Mixture of peptides	

sequence pattern as described in Fig. 2. In the lysozyme polypeptide chain, only the sequence around the Trp^{123}-Ile^{124} bond fits the sequence pattern, but this sequence probably is poorly accessible due to a disulfide bridge linking Cys^{127} (the potential P_4' position) to Cys^6. Furthermore, the result of mild cleavage of fructose-1,6-bisphosphate aldolase by human cathepsin D, as reported by Offermann *et al.* (16), agrees well with the above sequence pattern. The human enzyme was found to cleave preferentially between Leu^{354} and Phe^{355} and in this case also, a basic residue was found at the P_5' position.

The amino acid sequence pattern at the preferential cleavage sites identified here is particularly likely to occur within an amphipathic α-helical segment of a protein. Such a helix, which is common on the surfaces of globular proteins, will by its nature tend to contain a sequence of 2 or 3 consecutive hydrophobic residues, directly followed by a charged residue in combination with another charged residue 3 positions or approximately one helical turn beyond the first. Indeed, the cleavage sites identified in myoglobin and cytochrome *c* are, with one exception, located in segments with an α-helical conformation in the protein crystals. Whether the same holds true for the cleavage sites in bovine serum albumin and the elongation factor Tu cannot be established, since the three-dimensional structures of these proteins have not been elucidated as yet. In view of the above, the possibility may be envisaged that cathepsin D prefers an α-helical folding of its substrate. This possibility is presently under investigation.

The results of the present study may be of relevance to the design of synthetic immunogens and vaccines that aim at T-cell activation. T-cells generally do not recognize intact protein antigens but rather fragments that are liberated during intracellular proteolysis; subsequently, these fragments are presented on the surface of antigen-presenting cells as a 1:1 complex with proteins that are encoded by the major histocompatibility complex, the so-called MHC-products (reviewed in 10). The results of ongoing studies in our laboratory indicate that at least in the case of myoglobin the protein fragments that are generated during processing within endosomes of antigen-presenting cells largely arise as a result of the proteolytic activity of cathepsin D.[1] In these studies, the *in vivo* processing of myoglobin within macrophages was mimicked in a cell-free digestion system containing partially purified endosomes from bovine alveolar macrophages. A preferential release of distinct fragments was observed. This endoproteolytic fragmentation by endosomal enzymes could be blocked completely by the addition of pepstatin, a powerful inhibitor of cathepsin D, and not with inhibitors of other classes of proteases. Moreover, identification of the myoglobin fragments that were produced revealed their close resemblance to the catheptic fragments identified in this study. Others have suggested a similar dominant role of cathepsin D in the processing of endocytosed human hemoglobin (8) and bovine serum albumin (9). So at least in these cases, and probably in many others too, cathepsin D-released fragments seem to be the dominant products of processing, and they are therefore likely to play a role in T-cell activation. This assumption is supported by the close overlap between the major catheptic fragments of myoglobin and pigeon cytochrome *c* and the immunodominant T-cell epitopes of these proteins (20–23, 29, 30).

Additional evidence supports the idea that in other cases also the activity of cathepsin D may be crucial in determining which peptide products arise from antigen processing and hence which protein segments may serve as T-cell epitopes. Two independent analyses of a large number of sequences that contain T-cell epitopes, irrespective of antigen or immunized host, have revealed that these sequences share common structural features. Berzofsky and colleagues (24) have reported evidence that the potency of an amino acid sequence to adopt an amphipathic α-helical conformation enhances the probability of such a sequence to be close to a T-cell epitope. Likewise, this probability is enhanced by the presence of a distinct amino acid sequence pattern. This pattern, as reported by Rothbard and Taylor (25), includes 2 or 3 consecutive hydrophobic residues, flanked by highly polar, often charged, amino acid residues or glycine. Both characteristics have been used successfully to predict the location of T-cell epitopes. It should be noted, however, that most experimental evidence does not point to a direct involvement of these characteristics in the recognition of protein fragments by T-cells. Therefore, the common structural characteristics of these sequences could be the result of a common factor during antigen processing such as the cleavage specificity of a processing enzyme. A general close proximity of T-cell epitopes to processing sites would, in such a case, explain the frequent occurrence of the above characteristics in the sequences of protein fragments that are used to identify T-cell epitopes. Below, evidence is discussed in support of this assumption.

When the generalized structural characteristics of T-cell epitope-related sequences are compared with the amino acid sequence pattern that is recognized by cathepsin D, the similarity is striking. The generic sequence pattern according to Rothbard and Taylor is almost identical to the sequence pattern recognized by cathepsin D (positions P_2 through P_2' in Fig 2). The preference of the enzyme for sequences with a potential to form an amphipathic α-helix has been discussed

TABLE II

Preferential cleavage sites in various protein substrates found upon mild cleavage by bovine spleen cathepsin D

Homologous amino acid residues at the various positions are enclosed by boxes.

Scissile bond	P_6	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'	P_5'	P_6'
Sperm whale myoglobin												
14–15	Leu	Val	Leu	His	Val	Trp	Ala	Lys	Val	Glu	Ala	Asp
29–30	His	Gly	Gln	Asp	Ile	Leu	Ile	Arg	Leu	Phe	Lys	Ser
111–112	Phe	Ile	Ser	Glu	Ala	Ile	Ile	His	Val	Leu	His	Ser
137–138	Asn	Lys	Ala	Leu	Glu	Leu	Phe	Arg	Lys	Asp	Ile	Ala
Pigeon cytochrome *c*												
67–68	Asp	Thr	Leu	Met	Glu	Tyr	Leu	Glu	Asn	Pro	Lys	Lys
82–83	Gly	Thr	Lys	Met	Ile	Phe	Ala	Gly	Ile	Lys	Lys	Lys
97–98	Ala	Asp	Leu	Ile	Ala	Tyr	Leu	Lys	Gln	Ala	Thr	Ala
Bovine cytochrome *c*												
67–68	Glu	Thr	Leu	Met	Glu	Tyr	Leu	Glu	Asn	Pro	Lys	Lys
82–83	Gly	Thr	Lys	Met	Ile	Phe	Ala	Gly	Ile	Lys	Lys	Lys
97–98	Glu	Asp	Leu	Ile	Ala	Tyr	Leu	Lys	Lys	Ala	Thr	Asn
Bovine serum albumin												
353–354	Phe	Leu	Gly	Ser	Phe	Leu	Tyr	Glu	Tyr	Ser	Arg	Arg
368–369	Tyr	Ala	Val	Ser	Val	Leu	Leu	Arg	Leu	Ala	Lys	Glu
430–431	Phe	Gln	Asn	Ala	Leu	Ile	Val	Arg	Tyr	Thr	Arg	Lys
445–446	Val	Ser	Thr	Pro	Thr	Leu	Val	Glu	Val	Ser	Arg	Ser
Elongation factor Tu												
35–36	Ala	Ile	Thr	Thr	Val	Leu	Ala	Lys	Thr	Tyr	Gly	Gly
311–312	Ser	Glu	Val	Tyr	Ile	Leu	Ser	Lys	Asp	Glu	Gly	Gly
331–332	Tyr	Arg	Pro	Gln	Phe	Tyr	Phe	Arg	Thr	Thr	Asp	Val

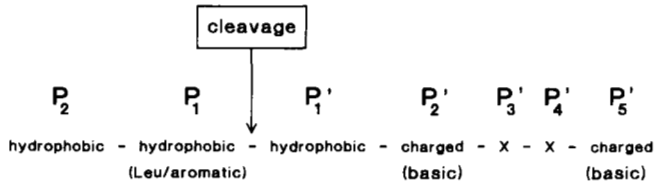

FIG. 2. **Amino acid sequence pattern common to sites that are preferentially cleaved by bovine spleen cathepsin D.** At the P_1 position, leucine and the aromatic residues are strongly favored; at positions P_2 and P_1', the hydrophobic requirements are less strict. A charged preferably basic residue is found at most P_2' and P_5' positions; at least 1 basic residue appears to be required by cathepsin D at either position.

above. In our view, the congruity between the structural characteristics that are recognized by cathepsin D and those that point to T-cell epitopes reflects the important role of cathepsin D in the processing of protein antigens within antigen-presenting cells. By catheptic cleavage of a polypeptide chain during processing, the polypeptide segments that flank the cleavage site will be made available for presentation to T-cells.

More specifically, the structural features recognized by cathepsin D have important consequences for the NH_2-terminal 5 amino acids of any resulting processing product (positions P_1'–P_5' in Fig. 2). In most cases, these NH_2-terminal amino acid residues will conform to the generalized sequence "hydrophobic-charged (preferably basic)-*X*-*X*-charged (preferably basic)." We postulate that this common feature among processing products provides a structural basis for their recognition by proteins that are encoded by the major histocompatibility complex on the surface of antigen-presenting cells. Evidence is accumulating that such recognition mainly depends on sequence patterns of amino acids with particular physicochemical characteristics rather than on specific amino acid entities (26–28). In line with the above postulate, it has recently been established for myoglobin that the NH_2-terminal 5 amino acids of two major catheptic fragments, 112–137 and 138–153, are indeed the exact target sites for such a recognition[3] (28).

At present, we are evaluating the T-cell-activating potential of peptides that are preferentially released from myoglobin by cathepsin D, directing special attention to the role of the NH_2-terminal sequence of such peptides.

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