REVIEW

Medical significance of cysteine protease inhibitors in mammalian secretory fluids

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Abstract : New cysteine protease inhibitors in human tears and milk and their medical significance are reviewed in this paper. As protective components against bacterial infection in the eyes, we detected four kinds of anti-bacterial proteins in normal human tears including lysozyme and three kinds of cysteine protease inhibitors. Using our reverse zymography of normal tears, three kinds of cysteine protease inhibitors were found to be 78kDa, 20kDa and 15kDa and were determined to be lactoferrin, Von Ebner's Gland (VEG) protein and cystatin S, respectively. All of them belong to the cystatin super family and VEG protein and cystatin S are well known cysteine protease inhibitors. The C-terminus area 17mer peptide, Y_{679}-K_{695}, of lactoferrin showed strong homology with a common active domain of the cystatin family and the synthesized peptide showed inhibition of cysteine proteases. Not only were disease-specific changes found in these inhibitor profiles, but also disease-specific new inhibitors in patients tears with certain autoimmune diseases. A 35kDa inhibitor, which was detected specifically in tears with Behcet's disease, an typical autoimmune disease, was determined to be a lacrimal acidic proline-rich protein based on the N-terminus sequence analysis. A65kDa inhibitor of tears with Harada's autoimmune disease was determined to be an Ig heavy chain V-III region. In addition, lactoferrin content in Harada's disease was very low. We found two cathepsin inhibitors in bovine milk using reverse zymography, namely lactoferrin and β-casein. The L_{133}-Q_{151}, in the human β-casein molecule is the active inhibitory domain. They may play an important role in antiseptic and anti-infectious functions. J. Med. Invest. 50 : 154-161, 2003

Keywords **:** *cystein protease inhibitor, human tears, reverse zymography, Behcet's disease, lactoferrin, milk, autoimmune disease*

INTRODUCTION

[I] Physiological roles of cysteine proteases.

Cysteine proteases, cathepsins, play an essential role in maintaining life in all living organisms. Cysteine proteases are synthesized in bound ribosomes and secreted from the trans-golgi, and then translocated into two ways, one is targeted into lysosomes and the other is secreted to the outside of cells through the secretion vesicles. Cathepsins located in lysosomes mainly play a role in protein catabolism *via* autophagy and heterophagy. On the other hand, the secreted cathepsins play a role in the processing of various biological active proteins, such as the processing of cytokines, hormones and inflammatory peptides. About 10 kinds of cathepsins have been reported and they have different functions and different catalytic properties. They play roles not only in protein catabolism, but also in the production of biologically active peptides by

Received for publication July 3, 2003 ; accepted July 24, 2003.

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their limited proteolysis, such as antigen processing to present to MHC class II (1 - 4), and also in the processing of various precursor proproteins (5). The hyper-function or low function of certain cathepsins results in specific metabolic error diseases. Therefore, intracellular regulation of cathepsin activities by their endogenous inhibitors *in situ* is very important.

[II] Physiological functions of endogenous cysteine protease inhibitors.

Cystatin α (A) in the skin and cystatin β (B) in the liver have been discovered by Katunuma's group and Turk's group, as the first endogenous proteinous inhibitors of cathepsins in mammals (6, 22). The cystatin family is classified into two groups, one group having low molecular weight with molecular weights of 10 -20 kDa, the others being high molecular weight inhibitors having a repeated peptide domain, such as kinin in the serum. Various kinds of cystatins are located in various organs and secretory fluids and they have three common binding domains with cathepsins in their molecules. Intracellular cystatins are principally located in the cytoplasm and various cystatins are also secreted into physiological secretory fluids such as tears, saliva or serum. However, the mechanism of action of cystatins in regulating the intralysosomal cathepsins has been relatively unclear. On the other hand, it was reported that the cystatins show strong bactericidal and virucidal functions due to the inhibition of cathepsins in bacteria. Katunuma *et al* reported that phosphorylated cystatin α located in the skin epidermis shows strong bactericidal action to Staphylococcus aureus V8 (7) and also Korant *et al.* reported that proliferation of poliovirus is strongly inhibited (8). Because, cysteine proteases in bacteria and viruses play important roles in maintaining their metabolism and life. Therefore, cysteine protease inhibitor play important roles in anti-septic and anti-infectious functions.

Recently, a different type of cathepsin inhibitor from the typical cystatin family was reported, by Hof *et al.*, that is, Von Ebner's Gland (VEG) protein in human tears(9). The VEG protein inhibits cathepsins considerably, however the VEG protein contains only one homologous active domain sequence with a common active site with the cystatin family, while the cystatin family possesses is three common binding sites. Since lactoferrin possesses only one binding domain, it may be considered a VEG protein-type inhibitor, although it belongs to the cystatin super family.

Furthermore, we found some novel types of new inhibitors from the cystatin family in human tears with certain autoimmune diseases.

A new method for detecting cysteine protease inhibitors in biological materials, named "reverse zymography", was used in this study.

MATERIALS AND METHODS

Inhibition analysis of the transferrin family against cysteine proteases

Recombinant rat liver cathepsins B, L, and C were used for inhibitory assay. Recombinant cathepsins K and S were expressed and purified according to the methods of Katunuma (10), Kopitar (11), and Bossard (12). The cysteine proteases were assayed using Z-Phe-Arg-MCA as a substrate for cathepsins L, B, S, K and papain, following the method of Barrett (13).

Synthesized peptide of near C-terminus 17 mer peptide of lactoferrin

The near C-terminus 17 mer peptide (Y_{679}-K_{695})of lactoferrin and 19mer (L_{142}-H_{160}) of human β-casein were chemically synthesized by Asahi Technoglass Co. (Chiba, Japan) with 95% purity. The synthesized peptide sequences were YEKYLGPQYVAGITNLK (Y_{679}-K_{695}) and LTDVENLHLPLPLLQSWMH (L_{142}-H_{160}).

Preparation of intramolecular peptides of β-casein

Bovine β-casein (250μg) in 100mM Tris-HCl buffer pH 8.5 was digested by lysylendopeptidase at 35°C for 16 hours. The digested sample was applied to HPLC, TSK gel DDS-80 Ts and eluted by a linear gradient using solvents of 0.1% TFA and 0.1% TFA in 90% acetonitrile. The main eluted peaks were used to assay the inhibitory activities and to determine the amino acid sequences.

Analysis of the N-terminus amino acid sequence

The N-terminus amino acid sequences of proteins and the isolated intramolecular peptides were determined with an HP G1005A protein sequencing system (Hewlett-Packard, Palo Alto, CA). After SDS-PAGE, the bands were transferred to a polyvinylidene difluoride membrane, and then subjected to amino acid sequence analysis using Majima's method (14).

Negative staining method of the SDS-PAGE gel

Negative staining of the gel was performed by the method of Fernandez, C. *et al.* (15). Samples of milk (10 -15 μl) were mixed with the same amount of sample buffer (0.125M Tris-HCl 4% SDS, 20% glycerol, 0.02% bromophenol blue pH 6.8). After the electrophoresis, the gels were incubated in a 0.2M imidazole solution

for 10 minutes. The incubation time could be modified depending on the acrylamide percentage. Then, the gels were transferred to a bath containing 0.2-0.3M zinc sulfate for1minute. For visualization, the protein bands were cut and washed with 2% citric acid to remove the staining solution. The gel pieces containing the protein bands were eluted and the eluates were used to check the inhibitory activity of the various authentic cysteine proteases.

RESULTS AND DISCUSSION

[1] Reverse zymography for the detection of cysteine protease inhibitors in natural materials

We developed a new detection technique for cysteine protease inhibitors in crude natural materials, and named it" reverse zymography"as shown in Fig.1. The principal of this detection method of protease inhibitors on SDS-PAGE is the reverse of that in usual zymography. The inhibitor samples were applied to special SDS gels co-coagulated with gelatin or without gelatin as the control. To digest the embedded gelatin, the gels were incubated with papain solution. The embedded gelatin and the coexistent proteins in the sample were digested. Gelatin preserved in the bands, in which the inhibitors were located, was stained with Coomassie brilliant blue. The SDS-PAGE was performed following the Laemmli method (16). Milk or tears were diluted with the same amount of a solution. After electrophoresis, the gel was removed, washed and transferred to a tray of 100 ml of acetate buffer containing papain and incubated at 37 °C for 10hours to digest the gelatin. The gel was washed with distilled water and then stained with Coomassie brilliant blue. The gels were then washed with destaining solution. Putative protease inhibitors were detected as blue bands on a clear background. The reverse zymography was compared with and without gelatin plates.

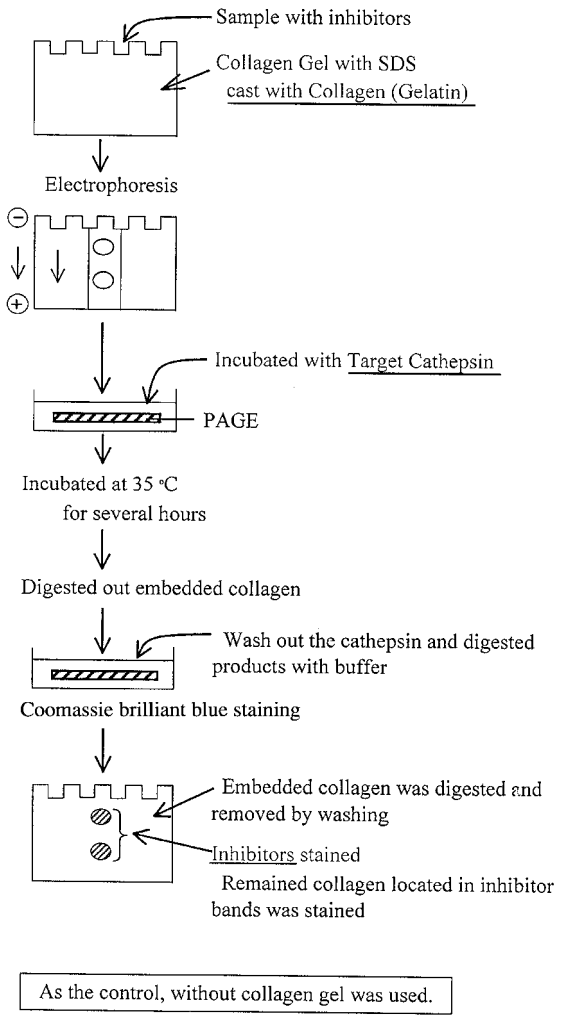

Fig.1. Schematic illustration of "Reverse Zymography" method for cysteine protease inhibitors.

[2] Basic demonstrations for detection of authentic protease inhibitors using the reverse zymography method

To demonstrate the corresponding inhibitors against the various target proteases using our reverse zymography, well-established authentic protease inhibitors and the pure corresponding proteases were employed. For example, the pairs for reverse zymography, cystatin C for papain, lactoferrin for papain and soybean trypsin inhibitor for trypsin, are demonstrated in Fig.2. After electrophoresis of the gelatin gel to which cystatin C or lactoferrin was applied, the gel was incubated with papain to hydrolyze the background gelatin. The washed gel was stained with Coomassie brilliant blue. Only the bands in which cystatin C or lactoferrin was located were stained at positions corresponding to 15 kDa or 78kDa, respectively, because the embedded gelatin in the inhibitor bands remained, as shown in Fig.2-B or Fig.2-C, respectively. Using the same method, soybean trypsin inhibitor was detected in the 25 kDa area using trypsin as the corresponding digestive protease to remove the embedded gelatin as Fig.2-A shows. The without-gelatin gels were used as their controls. We could selectively detected corresponding inhibitors to various target proteases by choosing various target proteases as digesting proteases of the embedded gelatin in the gel. Without-gelatin gels were used as the corresponding controls as shown in lanes 3, 5 or 7 in Fig.2. The contaminated proteins in the natural materials were also digested out to produce a clean background. The remaining gelatin in the inhibitor band was stained blue on a white background.

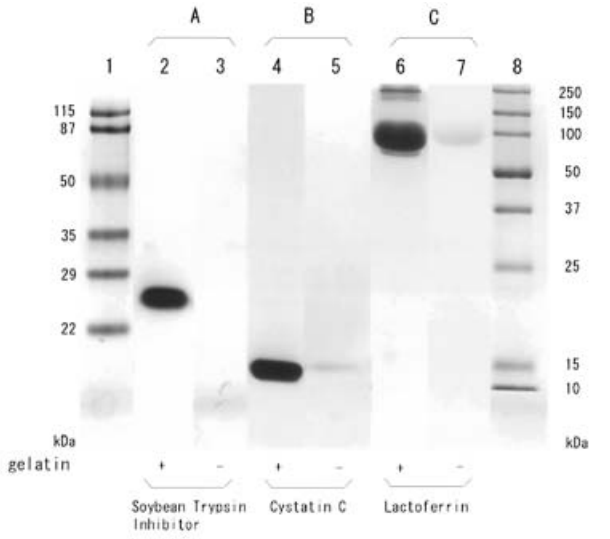

Fig.2. Specific detection of authentic protease inhibitors by reverse zymography.

Lanes1 and 8 are molecular markers. [A] Soybean trypsin inhibitor was applied in lanes 2 and 3, and embedded gelatin was digested out by trypsin; Lane2 is with-gelatin gel and lane3 is without-gelatin gel as the control. The remaining gelatin band in the soybean trypsin inhibitor band of 25 kDa is stained by Coomassie brilliant blue in lane 2. [B] Cystatin C was applied in lanes 4 and 5, and the embedded gelatin was digested out by papain; lane4 is with-gelatin gel and lane 5 is without-gelatin gel as the control. The remaining gelatin band in the cystatin C band of 15 kDa is stained in lane 4. [C] Lactoferrin was applied in lanes 6 and 7, and the embedded gelatin was digested out by papain; lane 6 is with-gelatin gel plate and lane 7 is without-gelatin gel plate as the control. The remaining gelatin in the lactoferrin band of 78kDa is stained in lane 6. The without-gelatin control gels in lanes 3, 5 and 7 are not stained.

[3] Disease-specific expression of new inhibitors in human tears

(1) *Inhibitory proteins of cysteine proteases in normal human tears*

More than 10 kinds of major protein components in normal human tears were detected using Coomassie brilliant blue staining of SDS-PAGE and as well as by the negative staining of the SDS-PAGE, as shown in Fig.3. To detect the bands of cysteine protease inhibitors in human tears, our reverse zymography of gelatinolysis inhibition to papain was employed. As shown in Fig.3, at least two different kinds of strong staining bands of 78kDa and 15kDa and very weak staining bands of 65kDa and 20kDa in normal tears were detected by the reverse zymography, although the comparative strengths showed some individual differences. And very weak 65kDa band was detected in a rare case of normal tears.

(2) *Identification and properties of normal tears inhibitors*

The structures of the three bands, showing strong inhibitory activity, were identified using amino acid sequence analysis of their N-terminus area and/or the sequences of their intramolecular peptides. The

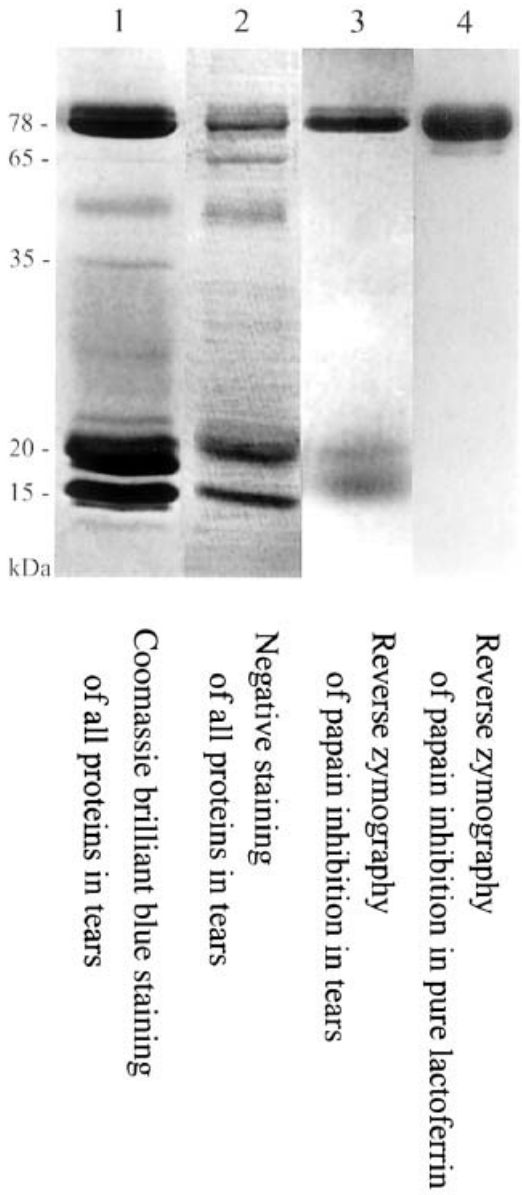

Fig.3. Reverse zymography of normal human tears.

The same amount of normal human tears were applied in lanes 1, 2 and 3. Lane1 ; normal tears was stained by Coomassie brilliant blue. Lane 2 ; normal tears was stained by negative Z staining method. Lane 3 ; normal tears was stained by reverse zymography method. Three main inhibitor bands were detected in78kDa of lactoferrin, 20 kDa of Von Ebner's Gland perotein and 15 kDa of cystatin S. Lane 4 ; recombinant lactoferrin was stained in 78 kDa band.

20 kDa inhibitor was identical to VEG protein based on the amino acid sequence analysis (8), and the band was cross-reacted with polyclonal antibody against anti-19 mer of N-terminus peptide, L_{21}-A_{39}(chemically synthesized), of the VEG protein molecule. The VEG protein was reported by Hof *et al.* to be a member of the cystatin super-family (8). The 15kDa inhibitor was estimated to be cystatin S, which is known to be a member of the cystatin family in saliva based on the molecular weight, the inhibitory profiles and the cross-reactivity with anti-cystatin S antibody (17). The 78 kDa

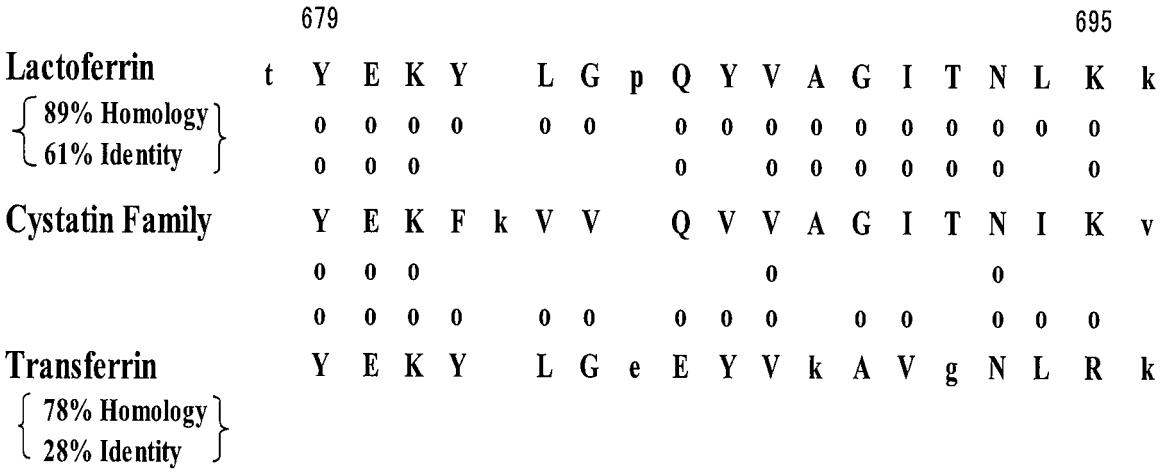

Fig.4. Homology of lactoferrin sequence near the C-terminus showing high frequency amino acids of common active site of the cystatin family.

Peptide sequence, Y_{679}-K_{695}, of lactoferrin showed 89% homology and 61% identity with that of active site of the cystatin family and transferrin sequence of the corresponding part had 78% homology and 28% identity with that of the cystatin family.

band inhibitor was determined as being a lactoferrin from the N-terminus sequence (20). The natures of the 78kDa, 65kDa, 20kDa and 15kDa bands were finally determined as lactoferrin, Ig heavy chain-V-III region, VEG protein and cystatin S, respectively, based on their amino acid sequence analysis. As shown in Fig.4, the near C-terminus peptide Y_{679}-K_{695}, of the lactoferrin molecule showed a strong homologous sequence with a common active site (binding site) of the cystatin family. In practice, this domain peptide synthesized chemically showed considerable inhibition to various cysteine proteases. Since the inhibition kinetics of lactoferrin to papain is of a non-competitive type, it is suggested that the lactoferrin does not compete with the synthetic substrate of papain. Authentic lactoferrin and β-casein were not degraded after incubation with papain using SDS-PAGE. Lactoferrin has been known to possess bacteriostatic action(18, 19), but the mechanism has not been determined. We clarified that the inhibition of cysteine proteases by lactoferrin must play a major role in exhibiting bactericidal and anti-septic functions, due to the strong cysteine protease inhibition of bacteria and viruses (7, 8).

(3) *Characteristic changes in the inhibitor profiles in pathological tears and detection of new disease-specific inhibitors in tears with specific autoimmune diseases*

The cysteine protease inhibitor profiles in tears of certain diseases showed characteristic changes from those of normal tears, and furthermore, novel disease-specific inhibitors were found in some autoimmune diseases (21). Characteristic reddish bands of 31kDa stained strongly with Coomassie brilliant blue were detected specifically in all eight cases of Behcet's disease which is an autoimmune disease, as Fig.5 (b) shows, and the lactoferrin contents in these cases were relatively high. The N-terminus sequence of the 31kDa reddish band specifically detected in the cases of Behcet's disease was 100% identical with that of human lacrimal acidic proline-rich protein. Furthermore, the eluates of the 31 kDa band area of SDS-PAGE (not stained) in the tears with Behcet's disease showed about 50% inhibition of papain at the protein concentration of 10^{-6} M. The cysteine protease inhibition of this kind of acidic proline-rich protein family has not been reported before. In the tears from four cases with Harada's disease, which is a typical autoimmune disease, the 65kDa inhibitors were strong and the lactoferrin content was relatively weak compared with those of normal tears as shown in Fig.5 (c). The 65 kDa band inhibitor expressed strongly in the cases of Harada's disease tears was determined as being a human Ig heavy chain V-III region based on the N-terminus sequence analysis. The N-terminus 10 mer sequence of the 65kDa band inhibitor was 100% identical with the human Ig heavy chain V-III region sequence. These inhibitors may have a relationship with the pathogenesis of these autoimmune diseases. The Ig heavy chain of variable III region was secreted extensively in Harada's autoimmune disease tears. Quantitative changes in the typical patterns of these inhibitors in specific eye diseases are compared by a scanning densitometry method in Fig.5.

Characteristic changes in these inhibitor contents and the expression of disease-specific inhibitors were found in Behcet's disease and Harada's disease. These unique changes in cysteine protease inhibitors in tears with specific autoimmune diseases may not only lead to the elucidation of their pathogenesis, but also be useful for differential diagnosis. The autoantigen of

Behcet's diseases is still unknown. It could be speculated that the proline-rich proteins are possible candidate of specific autoantigens that induce Behcet's disease, because these proteins belong to the collagen family.

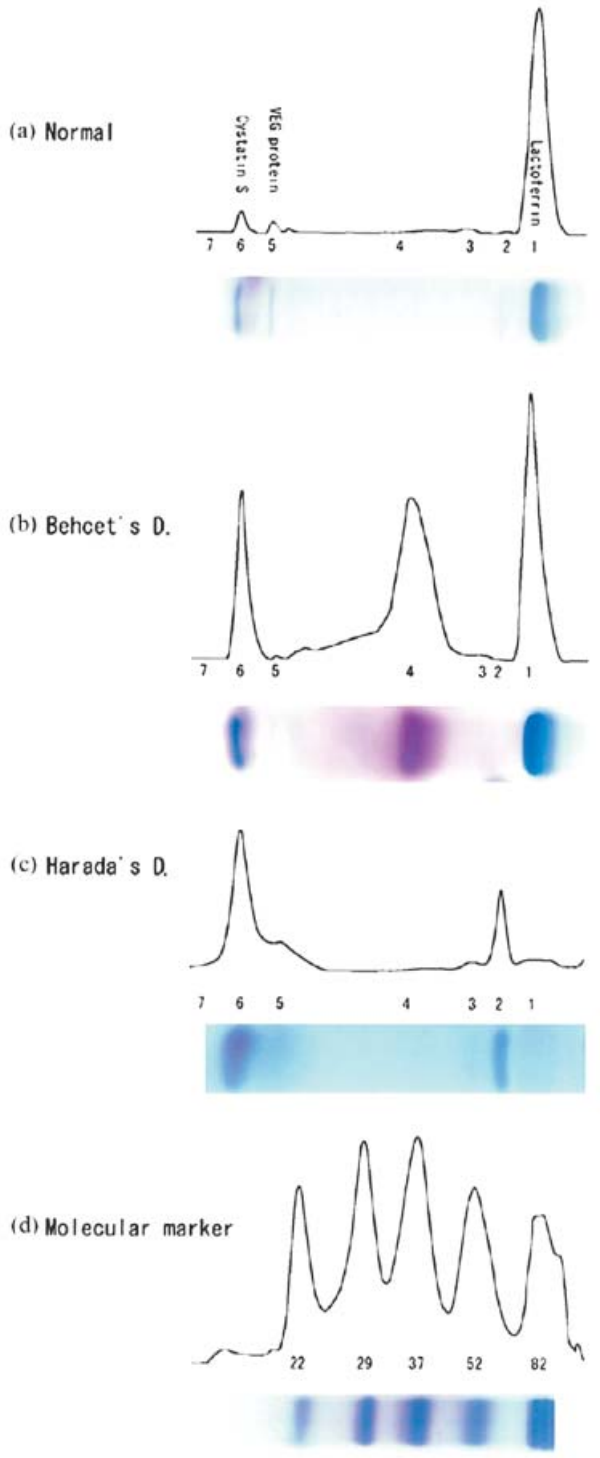

Fig.5. Disease-specific inhibitor patterns in typical cases of specific eye diseases were demonstrated quantitatively using the gel scanning method of reverse zymography.

Panel (a): normal tears. Panel (b): tears with Behcet's disease. Panel (c) : tears with Harada's disease.

No.1 band of 78kDa is lactoferrin. No.2 band of 65kDa is Ig heavy chain V-III region. No.4 reddish band of 31kDa is lacrimal acidic proline-rich protein of Behcet's disease-specific inhibitor. No.5 band of 20 kDa is VEG protein. No.6 band of 15 kDa is cystatin S.

[4] Lactoferrin and β-casein in mammalian milk as cysteine protease inhibitors

(1) Detection of lactoferrin and β-casein as cysteine protease inhibitors in human and cow milk

Human and cow milk were found to contain two cysteine protease inhibitors, namely lactoferrin and β-casein, using our reverse zymography for papain inhibition. The main inhibition bands in cow and human milk were found with apparent molecular weights of 78 kDa and 35kDa, which showed the same migration with recombinant lactoferrin and β-casein on their SDS-PAGE, respectively, as shown in Fig.6. Lane1shows all the proteins in milk using normal SDS-PAGE staining with Coomassie brilliant blue. Lane 2 shows the papain inhibition bands due to 78kDa of lactoferrin and 35kDa of β-casein in milk using reverse zymography, and lane 3 shows the control without the gelatin plate. Lanes 4 and lane 5 show reverse zymography of recombinant lactoferrin and lanes 5shows the control without the gelatin plate. Reverse zymography of recombinant human β-casein is shown in lanes 6 and 7, and lane 8 is the control without the gelatin plate. Lactoferrin and β-casein are the major inhibitors of cysteine proteases in mammalian milk.

The 78 kDa and 35 kDa staining bands of human milk were identified as lactoferrin and β-casein based on analysis of their N-terminus sequences. The N-terminus 10 mer sequence of the 78kDa band was completely identical to that of lactoferrin and the N-terminus 15mer sequence of the 35kDa band was completely identical

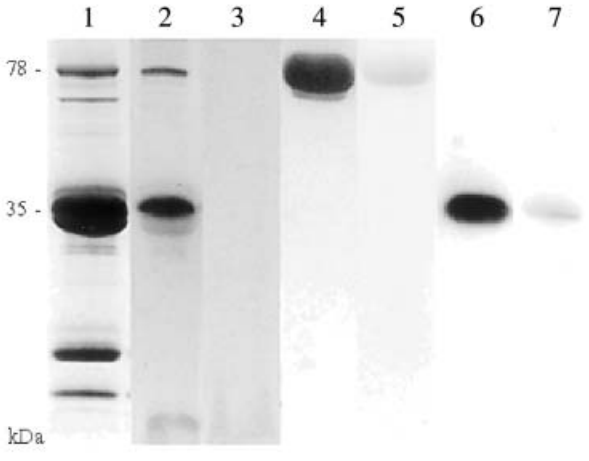

Fig.6. Detection of papain inhibitors in cow milk using reverse zymography of gelatinolysis inhibition.

Lane1 shows all the proteins in cow milk stained by Coomassie brilliant blue. Lanes 2 and 3 show reverse zymography of cow milk and lane 3 shows that without the gelatin plate as the control. Lanes 4 and 5 show reverse zymography of authentic lactoferrin and lane 5shows that without the gelatin plate as the control. Lanes 6 and 7 show reverse zymography of authentic β-casein and lane 8 shows that without the gelatin plate as the control.

with that of human β-casein. Furthermore, the eluates from the 78kDa band and the 35kDa band of the negative staining SDS-PAGE gel showed the inhibition of various cysteine proteases.

(2) Inhibition characteristics of human β-casein to cysteine proteases

β-Casein inhibited papain completely at 10^{-6} M. The inhibitory specificities of β-casein to various cysteine proteases were tested. β-Casein inhibited papain strongly and inhibited cathepsin L weakly at 10^{-5} M, but cathepsin B was not inhibited at10^{-5} M. However, we could not find homologous domain in the β-casein molecule with a common active site sequence of the cystatin family. Therefore, the inhibition mechanism must be different from that of cystatin. The inhibition mode of human β-casein to papain showed sigmoidal allosteric inhibition kinetics. The inhibition kinetics of human β-casein showed a second order sigmoidal curve to the substrate and the reciprocal plot between1/v and1/$[S]^2$gave a straight-line. A Hill constant was calculated as n=2.4using the Hill equation of log (v/V_m-v)= n log [S]-log K_m (V_{max}= 9,000 U and K_m=0.0079). The hydrolyzed products of bovine β-casein by lysylendopeptidase showed about the same inhibition as that of intact β-casein. The product peptides by the digestion were separated using reverse-phase HPLC, the papain inhibitions of these main peptides were assayed and the inhibitory peptide sequences were determined as LTDVENLHLPLPLLQSWMH (L_{142}-H_{160}) in bovine β-casein and LTDLENLHLPLPPLPLLQPLMH (L_{133}-Q_{151}) in human β-casein. Both peptide sequences showed 79% identity and 84% homology to each other. The synthesized peptide of L_{133}-Q_{151}in human β-casein showed significant inhibition to papain, and the peptide showed 68% inhibition at 10^{-5}M and 100% inhibition at 10^{-4}M, but the other parts of the separated peptides showed no inhibition. β-Casein is not only a nutritional protein source, but also plays a role as a cysteine protease inhibitor. The biological roles of cysteine protease inhibitors of lactoferrin and β-casein in mammalian milk are important from medical aspects. One of the important functions of lactoferrin and?-casein in milk may be to exert inhibitory effects to cysteine proteases of bacteria and viruses to play a role in anti-infectious and anti-septic functions (7, 8).

These contents were published in the following original papers.

1)A. Ohashi, E. Murata, K. Yamamoto, E. Majima, E. Sano, Q. T. Le and N. Katunuma: New functions of lactoferrin and β-casein in mammalian milk as cysteine protease inhibitors., *BBRC* **306**, 98-103 (2003). 2)Q. T. Le, E. Sano, A. Ohashi, E. Murata, H. Shiota, M. Ishimaru, K. Yamamoto, E. Majima, S. Isemura, and N. Katunuma : Disease-specific cysteine protease inhibitors in tears with autoimmune-diseases. (2003) *in preparation of Archives of Ophthalmology.* 3) N. Katunuma, A. Ohashi, E. Sano, E. Murata, H. Shiota, K. Yamamoto, E. Majima and Q. T. Le : New cysteine protease inhibitors in physiological secretory fluids and their medical significances., *Advances in Enzyme Regulation* **43**, 393-410 (2003).

ACKNOWLEDGEMENTS

Many thanks to Miss Rika Takahashi for her valuable help in preparing this manuscript.

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