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Biochemical and Biophysical Research Communications 306 (2003) 98–103
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New functions of lactoferrin and β-casein in mammalian milk as cysteine protease inhibitors☆

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Received 3 May 2003

Abstract

We found new inhibitory function of lactoferrin and β-casein in milk against cysteine proteases using reverse zymography. The inhibition of cathepsin L by lactoferrin was strongest and the inhibition kinetics were of a non-competitive type. Heat denatured lactoferrin lost the inhibitory activity completely, therefore the tertiary structure is essential to show the inhibition. Native lactoferrin was not degraded by papain during the assay condition. The intramolecular peptide, Y_{679}–K_{695}, of lactoferrin is an active domain and the synthesized peptide inhibited cysteine proteases. The Y_{679}–K_{695} peptide showed 90% homology with the sequences of a common active site of cystatin family. β-Casein and the active domain, synthesized L_{133}–Q_{151}, peptide inhibited cysteine proteases. Lactoferrin and β-casein in milk might play a role in antiseptic and antiinfectious functions due to cysteine protease inhibition of bacteria and viruses.

Keywords: Lactoferrin; β-Casein; Cysteine protease inhibitor; Milk; Cystatin; Reverse zymography

Mammalian milk contains large amounts of lactoferrin and β-casein. Lactoferrin is known to be a ferric-ion carrier and widely distributed in serum and mammalian milk [1]. One mole of lactoferrin binds to two moles of ferric ions and holo-lactoferrin releases the bound irons by acidic ammonium sulfate precipitation at pH 2.0 to make apo-lactoferrin. The biological function of lactoferrin is to carry ferric-ions and heme–iron can be synthesized via the lactoferrin–iron complex, but free-iron molecule cannot use for the synthesis of heme. It is also known that the lactoferrin shows strong bacteriostatic-action. However, the mechanism has been speculated to be due to the oxidative function of the bound iron molecules [2–4]. We discovered that the lactoferrin showed a strong inhibition of cysteine proteases. The near C-terminus 17 mer sequence of lactoferrin showed strong homology with the sequence of a common active domain of cystatin family, furthermore the 17 mer binding domain is exposed on the surface of lactoferrin [5]. Therefore, lactoferrin may consider to be a member of the cystatin super-family. The cysteine protease inhibition by lactoferrin and β-casein is a novel finding with important biological implications. It has been reported that cystatin α in skin showed bacteriostatic action against *Staphylococcus aureus* V8 by our group [6] and Korant et al. [7] also reported that cystatin C suppresses the growth of poliovirus. Therefore, lactoferrin and β-casein in milk might play a role in the protection from bacterial infection and antiseptic action due to inhibition of cysteine proteases of bacteria and viruses. Since the human digestive tract has no cysteine proteases, lactoferrin and β-casein should not disturb digestion of food proteins. The inhibition mechanisms of lactoferrin are discussed at the molecular level in this paper.

☆ *Abbreviations:* kDa, kilodalton; MCA, methyl-coumarylamide; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; V_m, maximum velocity; [*S*], substrate concentration.

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doi:10.1016/S0006-291X(03)00917-3

Materials and methods

Inhibition analysis of lactoferrin family against cysteine proteases. Rat liver cathepsins B, L, and C were purified as reported previously [8–10]. Recombinant cathepsins K and S were expressed and purified according to the methods of Inaoka et al. [11], Kopitar et al. [12], and Bossard et al. [13], respectively. 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 et al. [14].

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

Preparation of intramolecular peptides of β-casein. Bovine β-casein (250 μg) in 100 mM Tris–HCl buffer, pH 8.5, was digested with lysyl-endopeptidase at 35 °C for 16 h. The digested sample was applied to HPLC, TSK gel DDS-80Ts, and eluted with a linear gradient using solvents of 0.1% TFA and 0.1% TFA in 90% acetonitrile. The main eluted peaks were used for assaying the inhibitory activities and the determination of the amino acid sequences.

Determination of N-terminus amino acid sequence. After SDS–PAGE, the bands were transferred to a polyvinylidene difluoride membrane, and were then subjected to amino acid sequence analysis. 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) using Majima's method [15].

Reverse zymography for detection of cysteine protease inhibitors in milk. We developed a novel detection method for cysteine protease inhibitors in crude natural materials and named it "*reverse zymography for cysteine proteases.*" The principal of this detection method of protease inhibitors on SDS–PAGE is the reverse of usual zymography. The inhibitor samples were applied to special SDS gels copolymerized with gelatin or without gelatin as the control. To digest the embedded gelatin, the gels were incubated with papain (31 U/ml) solution. The embedded gelatin and the other proteins in the sample were digested, thereby removing stainable proteins except in where the inhibitor bands were located. These preserved gelatin bands, in which the inhibitors were located, were stained with Coomassie brilliant blue. The SDS–PAGE was performed following the Laemmli method [16]. SDS–polyacrylamide slab gels were cast with substrate gelatin as follows [17]: slab gels were casted with 12.5% acrylamide, 0.3% bis-acrylamide, and 0.1% gelatin, or without gelatin as the control. The stacking gels contain 4% acrylamide and 0.14% bis-acrylamide. Milk (10–15 μl) was diluted with the same amount of a solution of 4.0% SDS, 20% glycerol, and 0.25 Tris–Cl, pH 6.8 (0.02% bromophenol). After the electrophoresis was completed, the gel was removed, washed, and transferred to a tray of 100 ml of acetate buffer at pH 5.5 containing 1 mg papain (31 U/ml) and was incubated at 37 °C for 10 h to digest the embedded gelatin. The gel was washed with distilled water and then stained with 0.025% Coomassie brilliant blue R250. The gels were then washed with destaining solution (40% methanol, 10% acetic acid, and 50% distilled water). Putative protease inhibitors were detected as the blue bands on clear background. The reverse zymography was compared with and without gelatin plates.

Negative staining method of SDS–PAGE. Negative staining of SDS–PAGE was performed by the method of Fernandez et al. [18]. Samples of milk (10–15 μl) were mixed with the same amount of sample buffer (0.125 M Tris–HCl, 4% SDS, 20% glycerol, and 0.02% bromophenol blue, pH 6.8). After the electrophoresis, the gels were incubated in a 0.2 M imidazole solution for 10 min. The incubation time could be modified depending on the acrylamide contents. Then, the gels were transferred to a bath containing 0.2–0.3 M zinc sulfate for 1 min. For visualization, the protein bands were cut and washed with 2% citric acid to remove the staining solution. The protein bands containing inhibitors were eluted and the eluates were used to check the inhibitory activity to the various authentic cysteine proteases.

Results and discussion

Detection of lactoferrin and β-casein as cysteine protease inhibitors in mammalian milk using reverse zymography

Two cysteine protease inhibitors in cow milk (also human milk, data not shown), lactoferrin and β-casein were detected using our SDS–PAGE reverse zymography for papain inhibition as Fig. 1 shows. The main two inhibitor bands in milk were detected with apparent molecular weights of 78 and 35 kDa, which showed the same migrations as recombinant lactoferrin and β-casein in the SDS–PAGE reverse zymography, respectively, as shown in Fig. 1. Lane 1 shows all proteins in milk using normal SDS–PAGE (without gelatin in the plate) stained with Coomassie brilliant blue. In lane 2, two main papain inhibition bands of 78 kDa of lactoferrin and 35 kDa of β-casein in milk and minor amount of cystatin clostrum [19] were detected using our reverse zymography for papain. Lane 3 shows the control of milk using the plate without gelatin. Lanes 4 and 5 show the reverse zymography of recombinant lactoferrin and lane 5 shows the plate without gelatin as the control. Reverse zymography patterns of recombinant human β-casein are shown in the lanes 6 and 7, and the lane 7 shows the control plate without gelatin. Lactoferrin and β-casein were the major inhibitors of cysteine proteases in mammalian milk, as Fig. 1 shows.

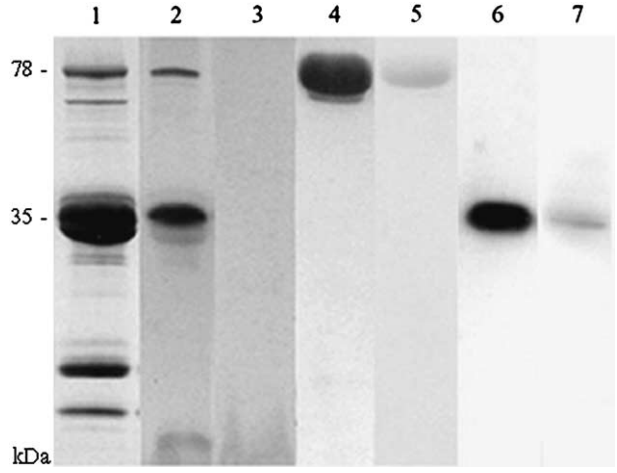

Fig. 1. Detection of papain inhibitors in cow milk using SDS–PAGE reverse zymography of gelatinolysis inhibition. Lane 1 shows all proteins in SDS–PAGE of cow milk stained by Coomassie brilliant blue. Lanes 2 and 3 show the reverse zymography of cow milk and lane 3 shows the control plate without gelatin. Lanes 4 and 5 show the reverse zymography of authentic lactoferrin and lane 5 shows the control plate without gelatin. Lanes 6 and 7 show the reverse zymography of authentic β-casein and the lane 7 shows the control plate without gelatin.

Identification of 78 kDa band of lactoferrin and 35 kDa band of β-casein

The 78 and 35 kDa staining bands of human milk in the reverse zymography were identified as human lactoferrin and human β-casein, respectively, based on the analysis of their N-terminus sequences, as shown in Figs. 2 and 3. The N-terminus 10 mer sequences of the 78 kDa band were completely identical with that of human lactoferrin and the N-terminus 15 mer sequences of the 35 kDa band were also completely identical with that of human β-casein. Furthermore, the eluates from 78 band and 35 kDa band of negative staining SDS–PAGE showed the same inhibitory profiles to various cysteine protease activities as those by recombinant lactoferrin and recombinant β-casein, respectively, as shown in Table 1.

Homology of a near C-terminus peptide in lactoferrin sequences with a common active domain structures of cystatin family

As shown in Fig. 2, the 17 mer sequences of Y_{679}–K_{695} in near C-terminus of lactoferrin molecule showed 90% homology and 60% identity with the sequences of a common active site (available high frequency amino acids) of cystatin family [21,22]. The corresponding part of transferrin sequence showed 82% homology and 35% identity [23]. Lactoferrin inhibits papain completely by 10^{-6} M, while transferrin inhibits papain at 10^{-5} M level.

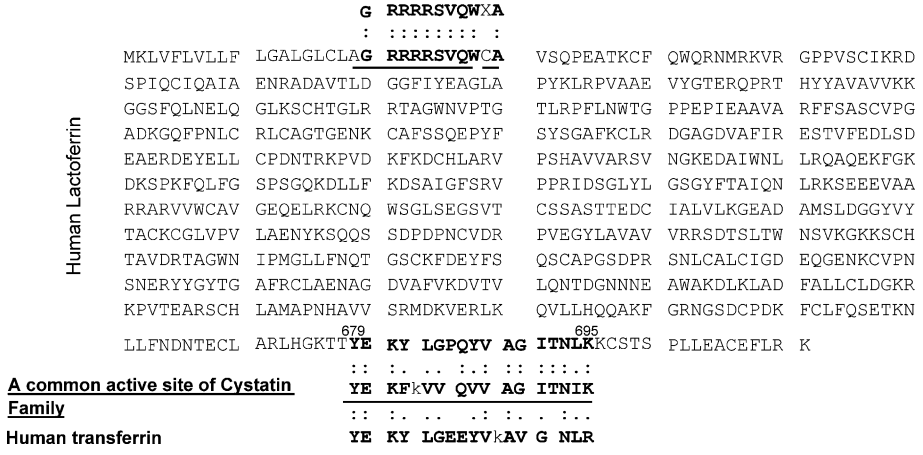

Fig. 2. Identification of N-terminus sequence of the 78 kDa inhibitor in human milk with lactoferrin and the sequence Y_{679}–K_{695} in lactoferrin with a common active site of cystatin family. The N-terminus 10 mer sequence of the 78 kDa inhibitor in human milk was completely identical with that of authentic human lactoferrin. The near C-terminus Y_{679}–K_{695} peptide sequence showed strong homology with a common active site sequence of cystatin super-family as shown in Fig. 3. The active inhibition peptide in human lactoferrin is indicated as an underlined sequence. The identical amino acids were illustrated two dot between (:) and the homology amino acids were illustrated one dot between (.).

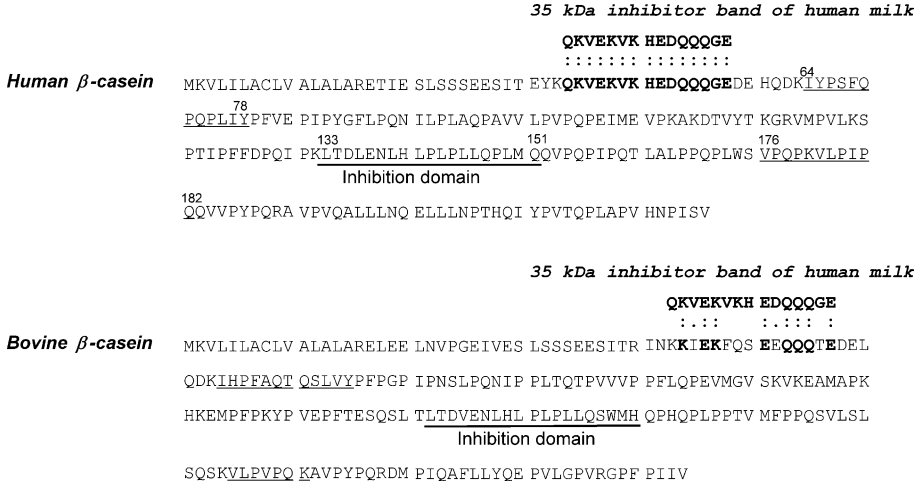

Fig. 3. Identification of N-terminus 15 mer sequence of 35 kDa inhibitor in human milk with β-casein. The N-terminus 15 mer sequence of the 35 kDa inhibitor in human milk was completely identical with that of human β-casein and showed also strong homology with that of bovine β-casein. The active inhibition domains in human and bovine β-casein molecules are indicated as an underlined sequence. Both 15 mer peptide sequences show 93% identity and 100% homology.

Table 1
Percent inhibitions of lactoferrin, active domain peptide of lactoferrin, or β-casein against various cysteine proteases

	Inhibitor concentrations (M)				
	10^{-7} (%)	10^{-6} (%)	10^{-5} (%)	10^{-4} (%)	
Lactoferrin					
Whole molecule	50	100			Cathepsin L
	0	90	100		Papain
		0	10	100	Cathepsin S
		0	20	100	Cathepsin B
			0	0	Trypsin
Synthetic peptide T_{679}–K_{695}		0	50	100	Cathepsin L
		0	10	50	Papain
				0	Cathepsin B
β-*Casein*					
Whole molecule	0	60	100	100	Papain
		0	40	100	Cathepsin L
		0	10	100	Cathepsin B

Furthermore, this active binding domain is located on the sulfate of the stereostructure of lactoferrin using the known X-ray crystallography 3D structure [5]. Therefore, lactoferrin in mammalian milk may consider to be a member of cystatin super-family.

Inhibition characteristics of lactoferrin to cysteine proteases

The inhibition profiles of various cysteine proteases by recombinant lactoferrin are shown in Table 1. Lactoferrin completely inhibited papain and cathepsin L at 10^{-6} M, and cathepsin B and cathepsin S were inhibited at 10^{-4} M. Cathepsin C and trypsin were not inhibited. In contrast, transferrin showed a weaker inhibition than that of lactoferrin, 10^{-4} M of transferrin was required to show a 50% inhibition of cathepsin L and papain, but cathepsin B and papain were not inhibited (data not shown). Both the holo-form and apo-form of lactoferrin (with and without ferric-ions) showed the same inhibition against these cysteine proteases. Therefore, the bound irons and the binding areas of the irons do not participate in the inhibition. Since the inhibition kinetics of lactoferrin to papain were of a non-competitive type, it is suggested that lactoferrin does not compete with the substrate of papain. Lactoferrin and the β-casein were not degraded during incubation with papain using the SDS–PAGE, although the papain activity was inhibited completely by 10^{-6} M of lactoferrin as shown in Fig. 4. Heat denatured lactoferrin lost the inhibitory activity to papain and was degraded by papain (data not shown). Therefore, the tertiary structure is essential to show the strong inhibition against cysteine proteases. Furthermore, the chemically synthesized 17 mer peptide (Y_{679}–K_{695}) of the C-terminus area of lactoferrin showed considerable inhibition of cysteine proteases (about 20 times weaker than that of complete molecule), as shown in Table 1. Cathepsin L was inhibited to 50% at 10^{-5} M of the peptide and papain was inhibited to 50% at 10^{-4} M of the synthetic peptide. The other parts of peptides which were prepared by lysylendopeptidase digestion did not show papain inhibition. Recently, a different type of cathepsin inhibitor from the typical cystatin family was reported by Hof et al. [20], that is,

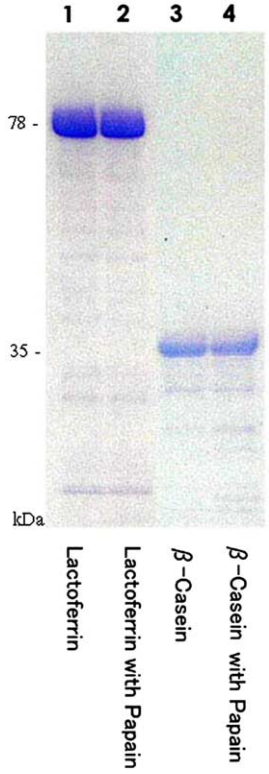

Fig. 4. Native lactoferrin and β-casein are not degraded by papain during the assay incubations. Lactoferrin (10^{-7} M) was incubated with 10^{-9} M of papain for 15 min. The papain activity was inhibited 100%. The reaction mixtures (without or with papain) were applied to SDS–PAGE and the lactoferrin was stained in lanes 1 and 2. The same experiments were done on β-casein in lanes 3 and 4.

Von Ebner's Gland (VEG) protein in human tears, which contains only one homologous sequence (less than 80% homology) in the N-terminus with a common active domain (QVVAGIT) of cystatin family, although typical cystatin family members have three common binding domains. However, the VEG protein showed considerable inhibition of cathepsins. Therefore, we have better to say that lactoferrin belongs to the VEG protein type inhibitor. Lactoferrin shows dual functions of iron carrier and cysteine protease inhibitor. The cysteine protease inhibitions of lactoferrin and β-casein in milk play an important role in protection from bacterial infection and antiseptic function. It is most important to know from biological aspects that the concentration of these inhibitors in natural milk is high enough to inhibit cysteine proteases of bacteria and viruses. Practically, the 50 times diluted natural milk inhibited the 10^{-9} M of papain completely, because lactoferrin and casein contents in milk are very high.

Inhibition characteristics of β-casein to cysteine proteases

β-Casein inhibited papain completely at 10^{-6} M. The inhibitory specificities of β-casein to various cysteine proteases are shown in Table 1. Papain was inhibited completely at 10^{-6} M of β-casein and cathepsin L was inhibited at 10^{-5} M, but cathepsin B was not inhibited at 10^{-5} M. However, any significant homologous sequence in the β-casein molecule with an active domain sequence of cystatin family is not found. Therefore, the inhibition mechanisms must be different from that of cystatin family. The β-casein was not degraded by papain at all, as shown in Fig. 4. The inhibition mode of human β-casein to papain showed sigmoidal allosteric inhibition kinetics, as shown in Figs. 5A and B. The inhibition kinetics of human β-casein showed a second order sigmoidal curve to the substrate concentration and the reciprocal plot between $1/v$ and $1/[S]^2$ gave a straight-line, as shown in Fig. 5B. The apparent Hill constant was calculated as $n = 2.4$ using the Hill equation of $\log(v/V_m - v) = n\log[S] - \log K_m$ ($V_{max} = 9000$ U and $K_m = 0.0079$).

Estimation of an inhibitory domain in β-casein molecule

The hydrolyzed products of bovine β-casein by lysylendopeptidase showed about the same inhibitory activity as that of the complete β-casein. The digested peptides were separated using reverse-phase HPLC and the papain inhibitions of these separated individual peptides were assayed. The inhibitory peptide sequences were determined as LTDVENLHLPLPLLQ (L_{142}–Q_{156}) in bovine β-casein and as LTDLENLHLPLPPLPLLQ (L_{133}–Q_{147}) in human β-casein as shown in Table 2. Both of the peptide sequences showed 93% identity and 100% homology. The synthesized peptide of bovine β-casein

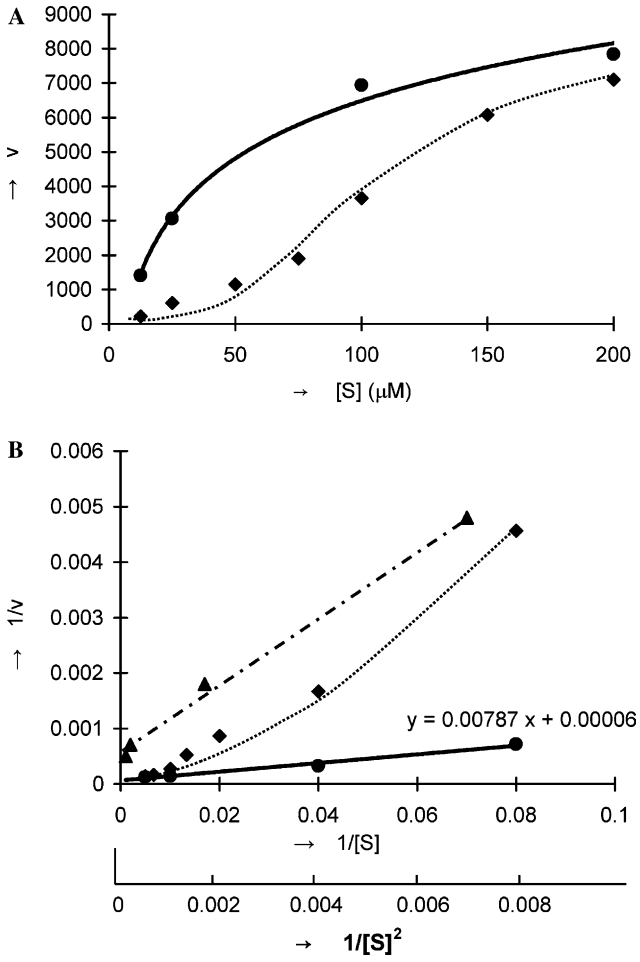

Fig. 5. Inhibition kinetics of β-casein to papain. (A) Substrate–velocity relationship. Without inhibitor: ●——●, solid line and with β-casein, ◆·····◆ dotted line. (B) Reciprocal plot of the substrate–velocity relationship using the Lineweaver–Burk plot. Line ●——● solid lines are without inhibitor. Line ◆·····◆ and line ▲–·–▲ are with 10^{-6} M of β-casein. (B) is reciprocal plot of $1/v$ to $1/[S]$. The symbols are the same as those in (A). Reciprocal plot between $1/v$ and $1/[S]^2$ gave almost a straight line; ▲–·–▲.

Table 2
Inhibition percentage to papain by various synthesized peptides in β-casein molecule

Peptides	Concentrations (M)		
	10^{-6} (%)	10^{-5} (%)	10^{-4} (%)
β-Casein (Human)	73	100	
L_{133}–Q_{151} (Human)	0	68	100
V_{176}–Q_{182} (Human)			0
I_{64}–Y_{75} (Bovine)			0

(L_{133}–H_{151}) itself showed significant inhibition to papain with 65% inhibition at 10^{-5} M and 100% inhibition at 10^{-4} M, and the other parts of the separated peptides by lysylendopeptidase digestion showed no inhibition as shown in Table 2. Therefore, these peptide domains of β-casein were estimated to be the active domains to bind

with cysteine proteases. β-Casein in milk was not only a nutritional protein, but also plays a role as a cysteine protease inhibitor.

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