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Proteolytic activity of bovine lactoferrin

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Abstract

Bovine lactoferrin catalyzes the hydrolysis of synthetic substrates (i.e., *Z*-aminoacyl-7-amido-4-methylcoumarin). Values of K_m and k_{cat} for the bovine lactoferrin catalyzed hydrolysis of Z-Phe-Arg-7-amido-4-methylcoumarin are 50 μM and 0.03 s^{-1}, respectively, the optimum pH value is 7.5 at 25 °C. The bovine lactoferrin substrate specificity is similar to that of trypsin, while the hydrolysis rate is several orders of magnitude lower than that of trypsin. The bovine lactoferrin catalytic activity is irreversibly inhibited by the serine-protease inhibitors PMSF and Pefabloc. Moreover, both iron-saturation of the protein and LPS addition strongly inhibit the bovine lactoferrin activity. Interestingly, bovine lactoferrin undergoes partial auto-proteolytic cleavage at positions Arg415-Lys 416 and Lys440-Lys441. pK_a shift calculations indicate that several Ser residues of bovine lactoferrin display the high nucleophilicity required to potentially catalyze substrate cleavage. However, a definitive identification of the active site awaits further studies.

Abbreviations: bLf – Bovine lactoferrin; hLf – Human lactoferrin Z-aminoacyl-AMC – *N*-α-benzyloxycarbonyl-aminoacyl-(7-amido-4-methylcoumarin); PMSF – Phenylmethanesulfonyl fluoride; Pefabloc – 4-(2-aminoethyl)-benzenesulfonyl fluoride; LPS – lipopolisaccaride.

Introduction

Lactoferrin is a mammalian iron binding protein present in external secretions, such as breast milk, tears, saliva, and vaginal secretions and in polymorphonuclear leukocytes. Lactoferrin has been shown to play a role in host defense mechanisms related to the non-immune defense system against pathogenic bacteria, fungi, and protozoa, both directly and through regulation of the inflammatory response (Brock 2002). Human lactoferrin has been recognized to cleave and to inactivate two colonization factors of non-typable *Haemophilus influenzae*, possibly attenuating the ability of this pathogen to cause localized mucosal illnesses such as otitis, hindering the ability of the bacterium to bind host epithelial cells and to form microcolonies (Qiu *et al.* 1998, Plaut *et al.* 2001). Remarkably, human lactoferrin has been proposed to belong to the serine protease family, cleaving IgA1 protease from *Haemophilus influenzae* at an arginine-rich region (Hendrixson *et al.* 2003). In the present study, we report direct evidence indicating

that bovine lactoferrin (bLf), like the homologous human protein, possesses trypsin-like activity. However, the catalytic efficiency towards synthetic substrates is very low. Thus the biological significance of this observation remains to be elucidated.

Materials and methods

Reagents: all the reagents were from Sigma-Aldrich, (St. Louis USA), unless otherwise indicated.
Bovine Lactoferrin (bLf) was extracted and purified from raw cow milk according to Sharma *et al.* (1999). Holo- and apo-forms were obtained according to Giansanti *et al.* (2002).
Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)
SDS-PAGE was performed on a 12.5% polyacrylamide gel, either in the presence or in the absence of 4% 2-mercaptoethanol (reducing or non-reducing conditions, respectively).

Western-blot analysis

The protein samples were subjected to SDS–PAGE and transferred to cellulose nitrate membrane (Millipore, Billerica, MA USA) using Mini Trans Blot (Bio-Rad) apparatus. Lysozyme was used as a negative control. The membrane was saturated with 3% Bovine Serum Albumin (BSA) in Tris Buffer Saline: Tris-HCl 10 mM containing NaCl 9 g/l SDS 0.01% (TBS) for 2 hrs at room temperature and washed four times with TBS. The membrane was then incubated with polyclonal anti-hLf anti-serum (1:500) (Sigma-Aldrich) overnight at room temperature and washed four times in TBS. The membrane was then treated with an anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:2000) for 2 hrs at room temperature. After several washes with TBS, the membrane was developed by the addition of 3,3-diaminobenzidine (0.6 mM), hydrogen peroxide (4.2 mM) and 4-chloro-1-naphtol (3.6 mM).

Enzymatic in-gel digestion

The electrophoretic bands containing protein fragments of interest were excised from Coomassie Blue-stained gel. Destaining, reduction, alkylation, and in-gel tryptic digestion were carried out according to Shevchenko *et al.* (1996). Briefly, destaining was achieved by rinsing the excised gel pieces in 100 mM ammonium bicarbonate containing 50% acetonitrile. Proteins contained in the gel pieces were treated with 10 mM dithiothreitol to reduce disulphide bridges and then with 50 mM iodoacetamide to alkylate the cysteine residues. Tryptic digestion of the reduced and akylated protein fragments contained in the bands excised from the gel was then carried out in 20 μl of 25 mM ammonium bicarbonate containing 0.1 μg of trypsin at 37 °C for 18 h.

MALDI-TOF analyses

Aliquots (0.5 μl) of the peptide mixtures generated by tryptic digestion of the SDS-PAGE separated protein fragments were analysed by MALDI-TOF mass spectrometry. About 100 fmol of peptide mixtures were mixed with α-cyano-4-hydroxycynnammic acid (10 mg/ml in 50% acetonitrile, containing 125 fmol/μl ACTH and 25 fmol/μl angiotensin as internal standards), deposited onto a MALDI sample probe and dried under ambient conditions. All mass spectra were generated on a MALDI-TOF mass spectrometer Voyager DE^{TM} PRO (Applied Biosystems), operating in the positive reflectron mode. Mass spectra were acquired from each sample by accumulating 100 laser shots and were calibrated using as internal standards the monoisotopic peak of angiotensin (m/z 931.5154) and of ACTH (m/z 2465.1989), so that the error on the experimental mass measurements is less than 30 ppm, and therefore, in perfect agreement with the theoretical molecular weight of the analysed peptides. All mass values are reported as monoisotopic masses.

Catalytic activity measurements

N-α-benzyloxycarbonyl-Phe-Arg-7-amido-4-methylcoumarin (Z-Phe-Arg-AMC), Z-Ala-Arg-AMC, Z-Arg-Arg-AMC, Z-Gly-Gly-Arg-AMC, and Z-Arg-AMC were from Sigma-Aldrich, (St. Louis USA). Z-Phe-Ala-AMC was from Enzyme system products (Livermore, CA, USA). The bLf catalyzed hydrolysis of Z-aminoacyl-AMC was determined at pH 7.0 (20 mM phosphate buffer) and 20.0 °C. In a typical experiment, the bLf concentration was 0.1 μM and the substrate concentration ranged between 1 and 100 μM. The bLf catalysed cleavage of substrates was followed spectrofluorimetrically at 460 nm with an excitation wavelength of 380 nm, monitoring the fluorescence signal due to the AMC leaving group. The amount of Z-Phe-Arg-AMC hydrolyzed was calibrated letting the enzymatic reaction to go to completion and measuring the total amplitude of the signal. Protease activity inhibition studies were carried out

by addition of 10 mM phenylmethanesulfonyl fluoride (PMSF) or 10 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefabloc, from Roche Diagnostic GmbH, Mannheim, Germany) to the reaction mixture. The catalytic properties of fully apo- and holo-bLf and the effects on catalysis of lipopolysaccaride (LPS) from *E. coli* serotype 0111:B4 (1 mg/ml Sigma-Aldrich, St. Louis USA) and L-arginine (2 mM; from Sigma) were also investigated.

Serine protease affinity column

A 'Hi-Trap' column derivatized with benzamidine (from Amersham-Pharmacia Biotech) was used as serine-protease affinity column. The column (1 ml bed volume) was previously equilibrated with 50 mM Tris-HCl pH 7.4 containing 0.5 M NaCl. bLf (10 mg ml^{-1}) was dissolved in the same buffer and loaded onto the column. The column was then washed with 10 bed volumes of the equilibration buffer. Fractions enriched with benzamidine-binding protein(s) (e.g. serine-like proteases) were then eluted with 5 bed volumes of 10 mM HCl (pH 2.0) containing 0.5 M NaCl.

Electrostatic potential and pK_a shift calculations of bLf

Hydrogen atoms were added to the three-dimensional structures of human (PDB code 1B0L, Sun *et al.* 1999), bovine (PDB code: 1BLF, Moore *et al.* 1997), buffalo (PDB code 1CE2, Karthikeyan *et al.* 1999), and mare (PDB code: 1B1X, Sharma *et al.* 1999) lactoferrin and the resulting structures were energy-minimized using CHARMM macromolecular mechanics package (Brooks *et al.* 1983) and the CHARMM22 parameters and force field (MacKerell *et al.* 1998).

Electrostatic potentials and pK_a shifts values were calculated using the program DelPhi (Nicholls & Honig 1991) which solves the finite difference Poisson-Boltzmann equation for molecules of complex shape by mapping the dielectric and charge distribution of the molecules in a three-dimensional grid. Atomic charges and radii were taken from the CHARMM22 parameter set (MacKerell *et al.* 1998). Values of 4 and 80 were used for the dielectric constant of the protein interior and solvent, respectively. A grid of $149 \times 149 \times 149$ points was used, yielding a final resolution of 2 grid units $Å^{-1}$. The effective pK_a of a given residue in a protein (pK_{eff}) is determined by the protonation state of all the ionizable residues. Thus, the calculation of pK_{eff} involves the determination of the ionization state of all the residues of the protein (see, Alexov 2003, and references therein). This is a complex and computationally demanding problem. For our scope, i.e. the determination of the polarization extent of Ser residues, a much simpler approach can be undertaken. All the ionizable residues (Asp, Glu, Arg, Lys, His) were considered in their standard protonation state at pH 7.0, and the effect of the electrostatic potential generated by the protein on the nucleophilic properties of Ser residues (i.e. the pK_a shift) was calculated using the equation: $\Delta\ pK_a = q \times \phi/2.303$ where ϕ is the electrostatic potential value on the Ser Oγ atom and q is the change in charge upon Ser deprotonation ($q = -1$) (Yang *et al.* 1993).

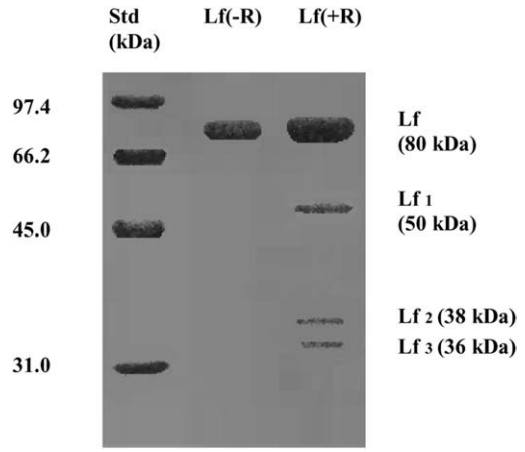

Fig. 1. SDS-PAGE of bovine lactoferin carried out in non-reducing (bLf - red) and in reducing (bLf + red) conditions. About 10 μg of bLf were incubated at 100 °C for 5 min in a denaturing solution containing 50 mM Tris/HCl pH 6.8, 8% glycerol, 1.6% SDS, 0.001% bromophenol blue, either in the absence or in the presence of 4% 2-mercaptoethanol (non-reducing or reducing conditions, respectively). The electrophoresis was performed on a 12.5% polyacrylamide gel. Protein bands were stained with Comassie Brilliant Blue. Bands of interest were cut from the gel and submitted to enzymatic in-gel digestion. LF: intact bovine lactoferrin; LF F_1: Fragment 1 (50 kDa); LF F_2: Fragment 2 (38 kDa); LF F_3: Fragment 3 (36 kDa).

Results

bLf autoproteolysis

Under non-reducing conditions, the SDS-PAGE analysis of bLf showed the presence of a single band with a molecular weight of 80 kDa (Figure 1, lane marked *bLf - red*), corresponding to intact bLf. More interestingly, three unexpected bands with apparent molecular weight of 50 kDa, 38 kDa, and 36 kDa

were observed in the SDS-PAGE analysis of bLf preparations when protein samples were treated with a reducing agent (Figure 1, lane marked *bLF + red*). The bands with apparent molecular weight of 50 kDa, 38 kDa, and 36 kDa accounted for less than 10% of the total protein and were all recognized by anti-Lf antibodies in a Western-blot analysis (data not shown). This indicates that proteolytic cleavage of bLf occurs, but, under non-reducing conditions, the bLf fragments remain linked by disulphide bonds. The four bands observed under reducing conditions were excised from the SDS-PAGE gel, submitted to in-gel tryptic digestion and the obtained peptide mixtures were analysed by MALDI-TOF-MS.

In order to unambiguously identify the sites of proteolytic cleavage of bLf, the analysis of the excised bands was carried out exploiting the mass-mapping strategy. The MALDI analysis of the peptide mixture obtained from the tryptic digestion of the 80 kDa protein band revealed specific signals originating from the intact protein, indicating that a large amount of the protein sample was not modified. The MALDI spectrum of the peptide mixture obtained from the 50 kDa protein band corresponded to the 1-415 bLf fragment, and no signals originating from the region 416-689 of bLf were detected. This result was confirmed by the MALDI-analysis of the peptide mixture obtained from the 38 kDa protein band, which corresponds to the 416-689 bLf fragment. Finally, the 36 kDa band corresponds to the 441-689 bLf fragment, indicating that a second cleavage site was located between Lys440 and Lys441.

bLf proteolytic activity

To determine whether the protein could be autocatalytically processed, the catalytic properties of bLf preparations with several synthetic substrates (Z-aminoacyl-AMC) were studied (see Materials and Methods). Fluorogenic substrates possessing an Arg at position P_1 were slowly hydrolyzed by bLf preparations, while those with Ala in position P_1 were not hydrolyzed. Z-Phe-Arg-AMC was the most susceptible to hydrolysis. In agreement with this primary bLf specificity, the addition of 2 mM L-arginine to the reaction mixture, competitively inhibited the bLf catalyzed hydrolysis of Z-Phe-Arg-AMC (data not shown). The proteolytic activity of bLf was observed in all the protein preparations obtained in our laboratory since 1995. However, the maximal proteolytic activity was observed in the most recent preparation, while the oldest

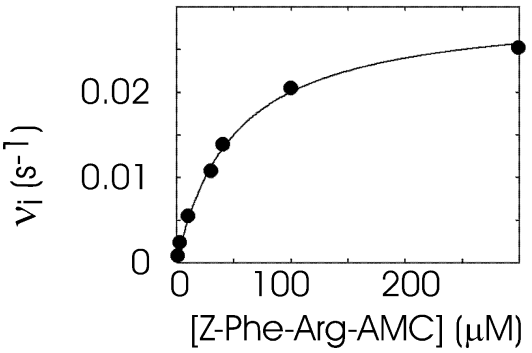

Fig. 2. Effect of substrate concentration on the rate of bLf catalyzed hydrolysis of Z-Phe-Arg-AMC. Experimental conditions: bLf 0.1 μM, Z-Phe-Arg-AMC 1-300 μM, Phosphate buffer 20 mM pH 7.0. Temperature = 20 °C. The continuous line was calculated from the classical Michaelis equation: $v_i = (k_{cat} \times [S])/(K_m + [S])$ with the following parameters $k_{cat} = 0.03\ s^{-1}$ and $K_m = 50\ \mu M$.

preparation, stored at −20 °C since 1995, showed the lowest activity (data not shown).

The bLf proteolytic activity is similar to that of serine-proteases. In particular, the bLf catalysed hydrolysis of Z-PheArg-AMC follows simple Michaelis-Menten kinetics and disappears when bLf is termally-denatured (Figure 2). Using apo-lactoferrin samples eluted from the serine-protease affinity column (see below), values of k_{cat} ($= 0.03\ s^{-1}$) and K_m ($= 50\ \mu M$) for the bLf catalysed hydrolysis of Z-PheArg-AMC were determined. The proteolytic activity displayed a pH optimum around pH 7.5 (data not shown) and either 10 mM PMSF or 10 mM Pefabloc irreversibly and almost completely inhibited bLf activity. bLf is usually 15–30% iron saturated when purified from milk. The highest catalytic activity of bLf was observed with the apo-protein, while iron-saturation of bLf almost completely inhibited the activity. As a control, incubation of the non-catalytically active, iron-saturated bLf with reducing agents and iron chelators (5 mM dithiotreitol and 1 mM EDTA) led to a time-dependent iron-desaturation of bLf and to restoration of the proteolytic activity (data not shown). Moreover, the addition of 1 mg ml^{-1} LPS, which is known to bind to clusters of positive charges present in both human and bovine lactoferrins (Appemelk *et al.* 1994, Van Berkel *et al.* 1997), strongly inhibits the proteolytic activity. On the contrary, no changes in the proteolytic activity were observed in the presence of 1 mM calcium, which is another small ligand for bLf (Rossi *et al.* 2002).

Table 1. Calculated pK_a shift of the hydroxyl group of the twelve serine residues strictly conserved in the N-lobe of selected mammalian Lfs. Numbering refers to hLf.

	pK_a shift			
Residue	Human	Bovine	Buffalo	Mare
12	**−6.5**	**−9.8**	**−6.6**	**−7.0**
114	−5.4	−4.0	−0.5	−3.6
154	−2.4	−3.1	−3.1	−1.6
156	−1.7	−0.4	−2.1	−0.1
184	**−5.0**	**−6.1**	**−6.7**	**−4.2**
193	+0.5	−4.7	−6.6	−1.5
252	−1.0	−0.1	+3.0	+0.3
259	−1.3	0.0	+0.5	+0.8
283	**−5.6**	**−4.6**	**−2.9**	**−6.1**
291	−2.7	−1.9	−3.9	−4.0
303	−5.9	−1.2	−0.3	−0.8
316	−2.1	−2.3	−2.2	−1.6

Binding of bLf to a serine protease affinity column

An affinity column for serine proteases was used to separate the protein with protease activity from bulk bLf. The 'Hi-Trap' affinity column is filled with agarose covalently coupled to benzamidine, a competitive serine protease inhibitor. Fractions eluted from the column were recovered and analysed. All the expected protein amount was recovered from washing (90% of the total recovered protein amount) and elution fractions. All the expected protease activity was found only in elution fractions. SDS-PAGE analysis of the elution fractions showed the presence of a single band at about 80 kDa, other proteins (e.g., contaminant proteases) were not detected. Western Blot analysis with anti-Lf antibodies revealed that the protein eluted from the column was indeed bLf (data not shown). Optical and fluorescence analysis of bLf eluted from the column did not show any marked spectral difference with respect to the native bLf from washing fractions (data not shown).

Analysis of surface serine residues

Multiple alignment of the amino acid sequence of the N-lobe of selected mammalian Lfs reveals the absolute conservation of twelve Ser residues (Table 1). Analysis of three-dimensional structures of the selected lactoferrins indicates that none of these Ser residues is part of a canonical serine protease catalytic triad. Thus, to test the hypothesis that the protease activity observed in bLf might be due to a 'non-canonical' serine protease active site, the polarization of the hydroxyl group of Ser residues strictly conserved in all mammalian Lfs analysed has been predicted through pKa shift calculations, using a finite difference Poisson-Boltzmann approach. As shown in Table 1, Ser12, Ser184, and Ser283 residues display a substantial pK_a shift in all Lfs analysed. Note that, the Ser259 residue, previously hypothesized to be part of the protease catalytic site in hLf (Hendrixson *et al.* 2003), is not characterized by a significant pK shift in any of Lfs analysed.

Discussion

It has been recently reported that hLf possess proteolytical activity towards two colonization factors of *Haemophilus influenzae*, and it has also been postulated that this activity decreases the patogenicity of this microorganism (Qiu *et al.* 1998, Plaut *et al.* 2001). In both colonization factors, the cleaved site appeared to be located in an arginine-rich region (Hendrixson *et al.* 2003). Present results clearly demonstrate that bLf also possesses autoproteolytic activity and proteolytic activity towards synthetic substrates. bLf appears to belong to the serine protease family as suggested by irreversible inhibition of the proteolytic activity by serine protease inhibitors such as PMSF and Pefabloc and by its reversible binding to benzamidine, though inactivation of cysteine proteases by the above mentioned inhibitors has also been reported (Barrett *et al.* 1998). Surprisingly, less than 10% of the lactoferrin molecules possess proteolytic activity, as indicated by the results obtained with the protease affinity results. However, no absorbance and fluorescence spectroscopic differences were observed in the bLf molecules displaying proteolytic activity, suggesting that there are no major conformational and/or chemical differences with the catalytically inactive pool of bLf molecules. On the other hand, both lactic acid bacteria and milk possess proteases (Barrett *et al.* 1998) and it cannot be excluded that bLf could strongly bind an acidic protease that copurifies and therefore, since the catalytic site has not yet been unequivocally identified, the presence of contaminant proteases cannot be positively ruled out.

In all the serine proteases so far characterized, the enzyme activity is dependent on the polarization of the hydroxyl group of the active site Ser residue due to a nearby His, Lys or *N*-terminal amino group (see,

Dodson & Wlodawer 1998, and references therein). Thus, in the effort to identify the Ser residue putatively responsible for the protease activity observed in human (Hendrixson *et al.* 2003) and bovine (present study) Lf we carried out pK_a-shift calculations on all the serine residues strictly conserved in mammalian lactoferrins (Table 1). The analysis was limited to the N-lobe of Lf since it has been reported that recombinant Lf N-lobe alone displays proteolytic activity (Qiu *et al.* 1998). However, this analysis did not yield unequivocal results, as Ser12, Ser183, and Ser284 residues (Table 1) showed comparable and substantial pK_a shifts, indicating altered pK_a values which would result in deprotonation and strong nucleophilicity of these residues at neutral pH. It is worthwhile to note that in our analysis Ser259, previously hypothesized to form a catalytic dyad with Lys 273 to generate the protease activity of human Lf (Hendrixson *et al.* 2003), did not show a significant pK-shift in any of the structures analysed. This result would suggest that Ser259 is unlikely to be the catalytic residue, at least without a large conformational rearrangement(s) of the neighbouring amino acid residues.

In conclusion, values of the catalytic constant for the bLf catalyzed hydrolysis of synthetic substrates seem too low to attribute much biological significance to this unexpected protein activity. However, synthetic substrates could be poor substrates as compared to the natural one and cysteine proteases inhibition by lactoferrin has been very recently reported (Ohashi *et al.* 2003).

Acknowledgements

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