A novel serum chitinase that is expressed in bovine liver[1]

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Abstract Chitinases are ubiquitous chitin-fragmenting hydrolases. They are synthesized by a vast array of organisms, including those not composed of chitin. Here, we describe a novel serum chitinase (chitin-binding protein b04, CBPb04), which is expressed in bovine liver. Although CBPb04 is secreted as an endocrine chitinase, it shows higher homology with human gastrointestinal tract exocrine chitinase (AMCase) than with macrophage endocrine chitinase (human chitotriosidase). This suggests that cows have a specific defense against chitin-containing microorganisms. CBPb04 mRNA is expressed in hepatocytes. This is the first report of a hepatogenic mammalian chitinase.

Key words: Chitin; Mammalian chitinase; Bovine serum; Liver; Innate immunity

1. Introduction

The immune response to microbial pathogens relies on both innate and adaptive components. The immediate, innate response is mediated largely by white blood cells, such as neutrophils and macrophages, cells that phagocytose and kill pathogens. Most pathogen cell-wall constituents, such as lipopolysaccharide, glucan, mannans, mannoproteins, and chitin, are recognized as foreign substances by endogenous pathogen recognition systems. Chitin is found in insects, crustaceans, and most fungi, but not in plants, vertebrates, or prokaryotes. One role of chitin, demonstrated recently in *Blastomyces dermatitidis*, is in anchoring and displaying a surface adhesin and virulence determinant [1]. Chitinases, however, are synthesized by a vast array of organisms, including organisms that do not produce chitin. The proposed role of plant chitinase is as a defense mechanism against chitin-containing organisms. Purified barley chitinase inhibits the growth of fungal hypha [2]. In mammals, human serum contains a chitinase, called chitotriosidase, and its mRNA is expressed in macrophages [3,4]. It has been proposed that human chitotriosidase plays a role in host defense responses similar to that of plant chitinases. Human chitotriosidase is the only mammalian endocrine secreted chitinase that has been identified and cloned. There have been many studies of human chitotriosidase, but few of other mammalian endocrine chitinases. In this study, we identified and purified a novel bovine serum chitinase, which is expressed in bovine liver. We also cloned the cDNA encoding the enzymatically active chitinase.

2. Materials and methods

2.1. Bovine serum

Bovine serum was prepared from the blood of a healthy Holstein cow. The serum was stored at −20°C before use.

2.2. Purification of CBPb04

To isolate bovine serum chitinase, called CBPb04, 3.5 l of bovine serum was applied to a chitin column (4×27 cm, chitin powder from crab shells, Nacalai Tesque). The column was washed at 1 ml/min with phosphate-buffered saline (PBS), and with 0.1 M EDTA and 0.1 M *N*-acetyl-D-glucosamine (GlcNAc, Nacalai Tesque), for eliminating C-type lectins and GlcNAc sensitive proteins, respectively, and then eluted with 500 ml of 0.1 M acetic acid at the same flow. The eluted peak was dialyzed against a saturated ammonium sulfate solution, and the resultant precipitate was collected by centrifugation at 10 000×*g* and 4°C for 30 min. The pellet was dissolved into the minimum volume of 100 mM Tris–HCl (pH 7.5). The clear supernatant was applied on a column of Superdex 200 (Amersham Pharmacia Biotech, Piscataway, NY, USA) equilibrated with 100 mM Tris–HCl (pH 7.5). The eluted fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The peak fractions were further purified with DEAE-TY650M (TOSOH). Unless specified otherwise, the protein concentrations were determined by UV measurement. The protein solutions were stored at −20°C until use.

2.3. Enzyme assay

Overall chitinase activity was determined using chitin azure as a substrate, suspended in McIlvaine buffer (100 mM citric acid, 200 mM sodium phosphate) at the indicated pH, as previously reported [5].

2.4. Amino acid sequence analysis and peptide mapping

The N-terminal sequence of purified CBPb04 was determined by automated Edman degradation using a PPSQ-21 protein sequencer (Shimadzu). To obtain internal peptides, the proteins were digested in gels by lysyl-end peptidase, extracted, and purified by reverse-phase chromatography using a μRPC C2/C18 column (Amersham Pharmacia Biotech). The sequences obtained were compared with those in the DDBJ. Peptide mapping was performed according to Goumon et al. [6]. The purified peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) (PerSeptive Biosystems).

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[1] The nucleotide sequence reported in this paper has been submitted to the DDBJ Nucleotide Sequence Database with accession number AB051629.

Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends

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2.5. Cloning of CBPb04

Total liver RNA was isolated from a Japanese Black bull using Trizol reagent (Gibco BRL, Grand Island, NY, USA). First strand cDNA synthesis was performed on 8 μg of total RNA using SuperScript™ II RNase H⁻ reverse transcriptase (Gibco BRL) and a dT18-tailed oligonucleotide primer. The first strand cDNA was used as the template for polymerase chain reaction (PCR) amplification with *Taq* DNA polymerase (Promega Co., Madison, WI, USA), and forward and reverse primers derived from an internal amino acid sequence. After PCR, the DNA fragment was purified, and subcloned into the pGEM-T easy vector (Promega Co.). DNA sequence analysis was performed using the Big Dye terminator method (PE Applied Biosystems). In order to amplify the missing 5′ and 3′ sequences, we performed 5′- and 3′-rapid amplification of the cDNA ends (RACE) using RACE kits from Gibco BRL.

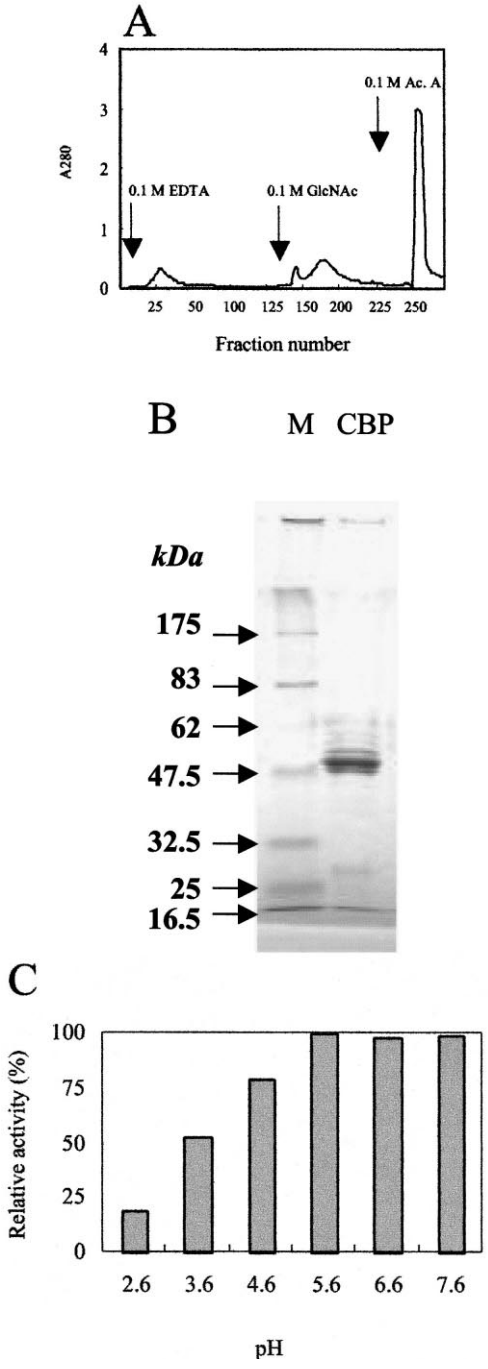

Fig. 1. Purification of CBPb04 and chitinase activity assay of the purified CBPb04. A: Affinity chromatogram. B: SDS–PAGE analysis of purified CBPb04. M, molecular weight standards; CBP, purified CBPb04. C: Chitinase activity assay of purified CBPb04.

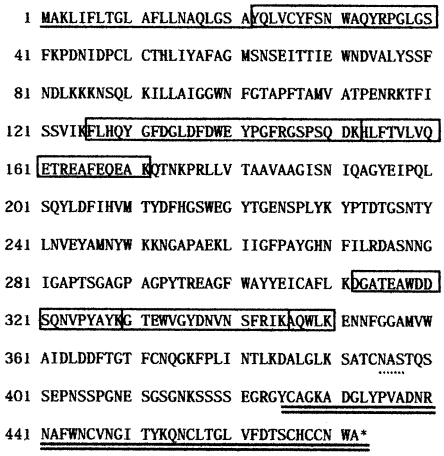

Fig. 2. Deduced amino acid sequence of CBPb04. The hydrophobic leader peptide (amino acids 1–21) is underlined with a single line. The putative chitin-binding domain (amino acids 425–473) is underlined with a double line. The N-linked glycosylation site is underlined with dots. The peptide sequences determined by the Edman method are boxed.

2.6. Northern blot analysis

Total RNA was separated in 1% agarose/formaldehyde gels, and transferred to nylon membranes (Amersham Pharmacia Biotech), which were hybridized with [α^{32}P]dCTP-labeled CBPb04 probe (position 503–960) and exposed to X-ray film (Kodak).

2.7. RT-PCR

To confirm the distribution of CBPb04 cDNA, an reverse transcriptase (RT)-PCR experiment was performed using *Taq* DNA polymerase and first strand cDNA as the template. The reaction was done using a CBPb04 cDNA-specific primer set.

2.8. Transient expression in 293T cells

The cDNA encoding CBPb04 was subcloned into the eukaryotic expression vector pCAGGS/MCS [7]. 293T cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS). FuGENE™ 6 Transfection Reagent (Roche) was used for the transfection experiments following the manufacturer's instructions. Recombinant CBPb04 in the medium was separated by affinity chromatography using a chitin column as described in Section 2.2.

2.9. In situ hybridization

In situ hybridization was performed as previously described [8]. Two non-overlapping antisense oligonucleotides, which are complementary to nucleotide residues 771–805 and 1074–1118 of the bovine CBPb04 cDNA, were used for in situ hybridization. These oligonucleotides were labeled with [^{35}S]dATP, using terminal deoxyribonucleotidyl transferase (Promega Co.). The cryostat sections were fixed, acetylated, and hybridized with the ^{35}S-labeled probes. Then, the sections were either exposed to Kodak BioMax MR for 1 week, or dipped in Kodak NTB2 nuclear track emulsion and exposed for 1 month.

3. Results and discussion

Chitin-binding protein was purified by affinity chromatography with a chitin column (Fig. 1A). One fraction eluted from chitin with 0.1 M acetic acid contained IgM, IgG, albumin, a 50-kDa protein, and a 20-kDa protein. This fraction was concentrated by dialysis against a saturated ammonium sulfate solution overnight and subjected to Superdex 200 gel

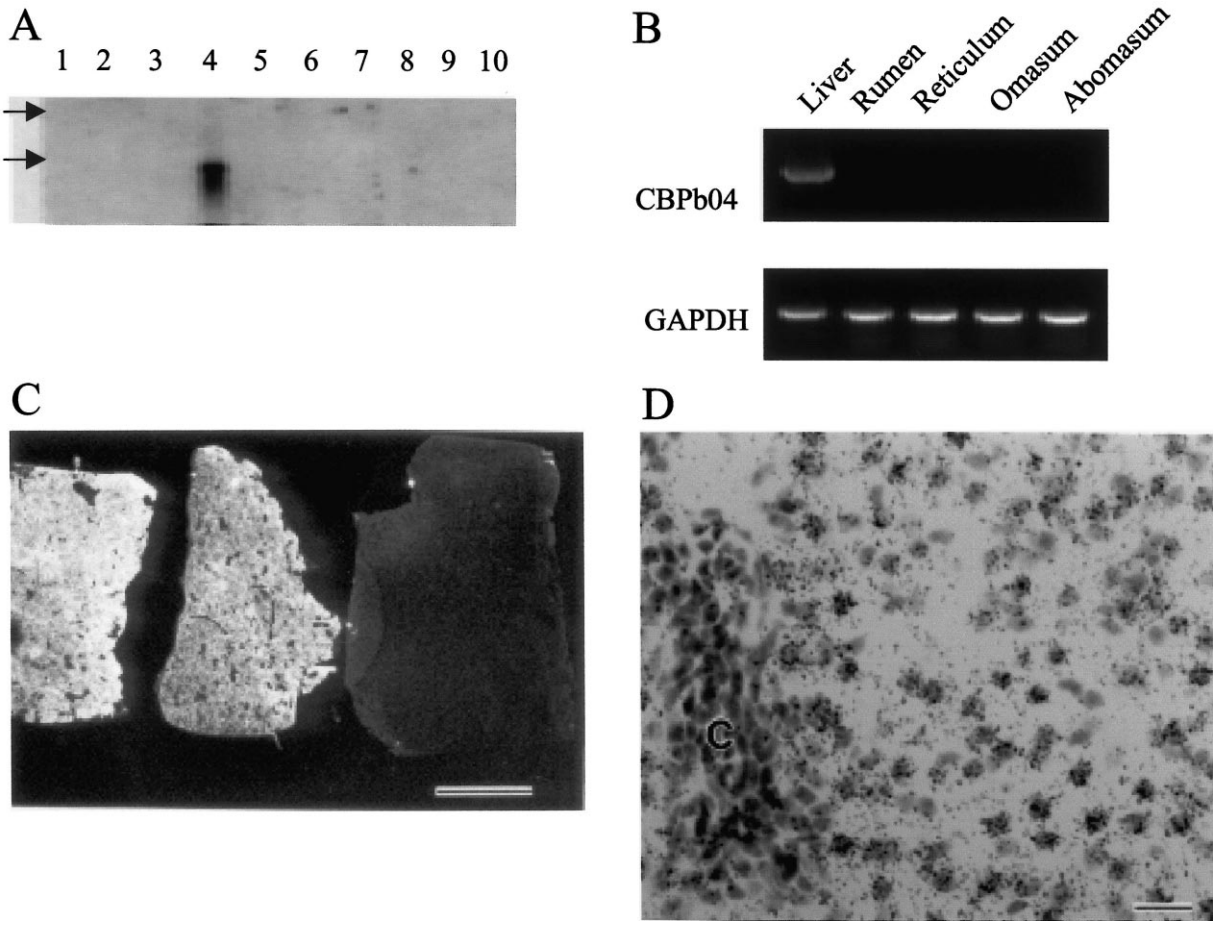

Fig. 3. The tissue distribution of CBPb04 mRNA. A: Northern blot analysis. 10 μg of total RNA was separated on an agarose gel as described in Section 2. The positions of the 28S and 18S rRNA bands are indicated with arrows. As a control for RNA loading, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used (data not shown). Lane 1, brain; lane 2, heart; lane 3, lung; lane 4, liver; lane 5, kidney; lane 6, spleen; lane 7, small intestine; lane 8, colon; lane 9, lymph node; lane 10, agmen peyerianum. B: RT-PCR. A CBPb04 cDNA-specific primer set was used to detect CBPb04 cDNA. As a control, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA-specific primer set was used. C: In situ hybridization for CBPb04 mRNA expression in the bovine liver (left and center panels) and mandibular gland (right panel). Hybridized sections were exposed to X-ray films. Intense signals, appearing as bright image, are found only in the liver. Bar = 5 mm. D: In situ hybridization (micro-autoradiography) showing CBPb04 mRNA expression in the liver. The signals are localized in all the hepatocytes, but not interlobular connective tissue (c) including blood vessels. Bar = 20 μm.

filtration. The fraction containing the ~50-kDa protein was further purified by anion exchange chromatography with a DEAE-TY650M column. CBPb04 was contained in a pass-through fraction, and appeared as three bands with a molecular mass of ~50 kDa (Fig. 1B). These proteins were identified as isoforms of the CBPb04 molecule by peptide mapping of lysyl-end peptidase digestion products (data not shown). The amino acid sequence of one internal peptide showed that CBPb04 possesses the highly conserved sequence of family 18 glycosyl hydrolases, which are also chitinases. Therefore, we investigated whether CBPb04 has chitinolytic activity. Chitinolytic activity was assayed using chitin azure as a substrate. CBPb04 showed chitinolytic activity, which was optimal at a pH between 5.6 and 7.6 (Fig. 1C). Therefore, CBPb04 is a bovine serum chitinase. Lundblad et al. [9] reported bovine serum chitinase activity in 1979, but there have been no reports in the literature since then. Their purification methods were very different from ours, so we cannot determine whether their chitinase was identical to CBPb04. Moreover, it is uncertain whether their chitinase was a chitotriosidase, because they did not report its primary structure.

To clarify the relationship between CBPb04 and human chitotriosidase, we obtained the cDNA sequence of CBPb04 from a combination of PCR products as described in Section 2. The nucleotide sequence of the cloned cDNA contained an open reading frame 1419 bp long, and the open reading frame encoded a protein with a characteristic N-terminal 21 amino acid endoplasmic reticulum signal peptide, immediately followed by the N-terminal sequence established for purified CBPb04. After removing the signal sequence, the cDNA encoded a predicted mature protein, 472 amino acids long (Fig. 2), with a calculated molecular weight of 49 954 and a p*I* of 5.26 (ExPASy Compute pI/Mw tool, Swiss Institute).

Overall sequence comparison of CBPb04 with human chitotriosidase showed 60.7% identity for the cDNA, and 51.4% identity for the deduced amino acids. Like human chitotriosidase, CBPb04 was predicted to contain an N-terminal catalytic domain of about 39 kDa, a hinge region, and a C-terminal chitin-binding domain. Analysis of the catalytic domain of bacterial chitinase was characterized by site-directed mutagenesis experiments in *Bacillus circulans* [10,11]. This revealed the importance of the ^{197}Asp, ^{200}Asp, ^{202}Asp, and ^{204}Glu residues

in the catalytic events of bacterial chitinase A1. This domain is also conserved in human chitotriosidase, but not in several mammalian chitinase-like proteins, which have no chitinase activity [12]. This domain is conserved in CBPb04, and expression of the cloned cDNA in 293T cells led to the secretion of an active chitinase (data not shown). This domain may also be essential for the chitinase activity of CBPb04. Tjoelker et al. reported that the C-terminal 49 amino acids of human chitotriosidase are necessary and sufficient for binding to chitin. Furthermore, they reported that mutation of ^{420}Cys, or any of the five cysteines within the binding domain, to serine renders it inactive [13]. CBPb04 also conserved all of these cysteines. CBPb04 might bind to chitin by this C-terminal domain, as does human chitotriosidase.

In spite of the similarities between CBPb04 and human chitotriosidase, it is inappropriate to consider CBPb04 as a bovine chitotriosidase. Screening the GenBank database using CBPb04 cDNA revealed high homology with TSA1902 (GenBank accession numbers AB025008 and AB025009) from a human lung cDNA library [14]. Independent of our study, Boot et al. recently reported a mammalian chitinase, AMCase, in the gastrointestinal tract; human AMCase (GenBank accession number AF290004) is almost identical to TSA1902 [15]. Sequence comparison of CBPb04 with human TSA1902/AMCase revealed 83.7% identity, which is much higher than that between CBPb04 and chitotriosidase (60.7%). Although the CBPb04 cDNA sequence showed high homology with TSA1902/AMCase, we observed CBPb04 mRNA expression only in the liver, and not in the abomasum, proventriculus (rumen, reticulum and omasum), or lung, where TSA1902/AMCase is expressed abundantly (Fig. 3A,B).

To our knowledge, human chitotriosidase is the only mammalian endocrine chitinase that has been cloned and characterized in detail. In our studies, however, chitotriosidase was not found in bovine serum and its cDNA was not detected in the spleen, where human chitotriosidase is abundantly expressed (data not shown). In situ hybridization analysis using two non-overlapping anti-sense oligonucleotide probes showed consistent labeling in all tissues examined. Intense signals for CBPb04 mRNA were found only in the liver, and not in the salivary glands, stomach, intestine, lung, or spleen (Fig. 3C). In the liver, CBPb04 mRNA expression was localized to essentially all hepatocytes (Fig. 3D). From a developmental perspective, since hepatocytes are derived from the primitive gut epithelium, CBPb04 might be more closely related to TSA1902/AMCase than to human chitotriosidase. In evolutionary terms, these results suggest that cows might have selected for secreting CBPb04 as an endocrine chitinase rather than as an exocrine chitinase, like AMCase. Since cows have abundant chitinolytic activity derived from lysozymes or chitinolytic microorganisms in the abomasum and rumen [16–18], a chitinase of host origin may not be required in the gastrointestinal tract.

It was recently reported that a recessively inherited deficiency in chitotriosidase is common [19–20]. About one of 20 individuals is completely deficient in enzymatically active chitotriosidase, and the prevalence of the deficiency suggests that chitotriosidase no longer fulfills an important defense function under normal circumstances. Therefore, it is possible that cows do not need chitotriosidase in serum. Alternatively, the serum chitinolytic activity of CBPb04 in cows may be sufficient for some innate immuno-response, such as protection against chitin-containing microorganisms. The ability to secrete CBPb04 as an endocrine chitinase might be an advantage in innate immunity, similar to that of plant chitinase. These findings suggest that studying endocrine and exocrine chitinases in cows and humans might be a key to characterizing the function of mammalian chitinases, which do not have a substrate in the mammal body.

In conclusion, we reported a novel serum chitinase that is expressed in bovine hepatocytes. This is the first report of a mammalian chitinase that is expressed in the liver. Although CBPb04 showed high homology with gastrointestinal tract exocrine secreted chitinase, CBPb04 was expressed only in the liver, and was secreted as an endocrine chitinase. These findings suggest that cows have a specific defense against chitin-containing microorganisms.

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