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Denatured human α-defensin attenuates the bactericidal activity and the stability against enzymatic digestion

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Abstract

α-Defensin is an antimicrobial peptide which plays an important role in innate immunity. Human defensin (HD)-5 is stored in the Paneth cells of the small intestine as a pro-form and is cleaved by trypsin, which is co-secreted from the Paneth cell granules. The mature HD-5 is protected from further digestion by the proteolysis enzyme. We generated both recombinant HD-5 and proHD-5, and the reduced form of each peptide in order to determine their physiological roles of the disulfide bonds. The reduced proHD-5 attenuated the bactericidal activity and the stability against the trypsin digestion. Human defensin was protected from the enzymatic degradation by disulfide bridges. We further purified the HD-5 with a disulfide variation in the small intestine of Crohn's disease patients. The HD-5 was sensitive to the trypsin treatment. These observations evidently predict that a defensin deficiency may be caused by a disulfide disorder in the disease.

Keywords: Enteric defensin; Paneth cells; Inflammatory bowel disease; Folding disorder

In the innate immune system of the gut, α-defensin, which is an endogenous antimicrobial peptide, is one of the major effectors against luminal bacterial invasion [1]. It is selectively produced by the Paneth cells which reside at the bottom of the crypt of the small intestine. A single crypt isolation technique from a mouse small intestine enabled us to elucidate the physiological roles of the Paneth cells, in which α-defensin is synthesized and secreted into the crypt lumen following bacterial stimuli, in order to provide antimicrobial activity [2]. The Paneth cells respond to several bacterial antigens independently of the toll like receptor (tlr)-4 expression, and the Paneth cell degranulation is enhanced by the CpG oligonucleotide via tlr-9 [3,4]. The calcium-dependent potassium channels are involved in the secretion of α-defensin from the Paneth cells into the lumen, and the cytosolic calcium influx is increased throughout the process [5].

The matrilysin gene knockout mouse, which is lethal against a *salmonella* infection, demonstrates that the processing of mouse α-defensin within Paneth cell granules by matrilysin is essential for the biological activity of the mouse defensin [6]. The activation process is completed within the Paneth cell granules before secretion, and thereafter the defensins are stored as mature forms in the mouse intestine [5]. In humans, two enteric α-defensins, HD-5 and HD-6, have been identified as gene products. The HD-5

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peptide is purified from the small intestinal tissue as a propeptide form [7–9]. Unlike mouse α-defensin, the HD-5 is stored as proHD-5 in the Paneth cell granules even though an endogenous digestive enzyme, trypsin is co-localized in the Paneth cell granules. After the granules are secreted into the crypt lumen, the proHD-5 is dominantly cleaved at the position of Arg^{62}–Ala^{63} by the trypsin [10].

We recently found that one of the natures of the mouse α-defensin family is resistance against the enzymatic cleavage by matrilysin and that this stability is due to the characteristic intra-molecular disulfide bonds [11]. Therefore, the post-translational processing of the pro α-defensin by the proteolysis should be properly regulated through the peptide maturation. However the functional roles of the disulfide bonds in human defensin are not yet understood.

In addition to the matrilysin gene knockout mouse, a gene ablation of a pattern recognition receptor also causes the lethal effect by a *Salmonella* infection. The *NOD2*-mutated mice are susceptible to bacterial infection and express a lesser amount of α-defensin [12]. Recently, a possible link between a mutation of pattern recognition receptor gene, *NOD2*/*CARD15*, and Crohn's disease (CD) was postulated [13,14], and this association was found in one-third of CD patients [15,16]. The NOD2 protein is abundantly expressed in the Paneth cells and thus innate immune deficiency could be associated with the etiology of CD [17,18]. This observation also supports the assumption that the Paneth cells may play a critical role in the innate immune defense and its related disorder.

Materials and methods

Generation of recombinant peptides and antibodies. The recombinant peptides were produced in *Escherichia coli* (*E. coli*) and then were purified as described [19]. The construct of the HD-5 specific sequences was kindly gifted by Ouellette AJ (University of California Irvine). The proHD-5 sequence was amplified from human small intestinal cDNA using primer sets (EcoRI-Met-proHD5-F: GAATTCATGGAGTCACTCCAGGAA and SalI-HD5-R: GTCGACTCATCAGCGACAGCAGAGTCT), and inserted in the pET28a vector (Novagen, Madison, WI). Those plasmids were then transformed into *E. coli* BL21(DE3)-CodonPlus-RIL cells (Stratagene, La Jolla, CA) for induction with 0.4 mM isopropyl-β-D-1-thiogalactopyranoside (Sigma–Aldrich, St. Louis, MO). The His-tag fusion peptides were purified with Ni–NTA Superflow (Qiagen, Valencia, CA). After 10 mg/ml cyanogen bromide (Sigma–Aldrich, St. Louis, MO) cleavage in 80% formic acid at room temperature overnight, the peptides were purified by C18 reversed-phase high performance liquid chromatography (HPLC; AKTAexplorer 10S, Amersham Biosciences, Piscataway, NJ).

The recombinant HD-5 peptide was conjugated with equimolar of ovoalbumin. The Rabbit was immunized twice by the conjugated peptide subcutaneously and the whole serum was harvested 7 weeks after injection.

Generation of linear peptide and trypsin cleavage. The recombinant proHD-5 was reduced by 5 mM DTT in 50 mM NH_4HCO_3 at 37 °C overnight. In order to obtain the reduced linear peptide, the reactant was acidified and applied to the HPLC separation in the C18 column at the gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid with a flow rate 2 ml/min for 60 min. The biochemical and physiological activity of the denatured or folded peptide was determined by acid urea–polyacrylamide gel electrophoresis (AU–PAGE) and an antibacterial assay.

Antimicrobial assay. Salmonella typhimurium PhoP-(strain CS015) were the target organisms for the standard bactericidal assays [20]. The quantities of the individual peptides were incubated with log-phase bacterial cells in a 50 μl buffer and the samples of the incubated mixtures were plated triplicate on semi-solid media after serial dilution. The surviving bacterial cells were counted as the surviving bacterial colony forming units (CFU).

Tissue preparation. Small intestinal specimens were surgically obtained from patients with either CD or colonic neoplasms, and then the fresh materials were frozen in liquid nitrogen and kept at −80 °C until use. Written informed consent was obtained from each patient. The samples were homogenized in 30% acetic acid with Polytron homogenizer (Polytron, Switzerland) in order to extract the protein. The supernatant which was clarified by centrifugation at 20,000 G for 60 min was lyophilized and dissolved in 1% acetic acid, then the concentration was measured with Bio-Rad Bradford protein assay reagent (Bio-Rad, Hercules, CA).

Western blotting. AU–PAGE followed by blotting and immunodetection were performed as described [21]. Briefly, 500 μg of proteins were lyophilized and incubated with or without 1 μg TPCK-trypsin (Pierce, Rockford, IL) in 50 mM NH_4HCO_3 at room temperature overnight and lyophilized again. The dried samples were loaded in 12.5% AU–PAGE gels and the resolved proteins were transferred to 0.2 μm pore nitrocellulose membranes (Invitrogen, Carlsbad, CA). The membrane was blocked by 5% skimmed milk, sequentially incubated with 1:2000 diluted anti HD-5 sera, horseradish peroxidase-conjugated anti rabbit IgG (1:20,000), chemiluminescent substrate (SuperSignal Wesr pico, Pierce, Rockford, IL) and then was developed using X-ray films (Kodak, Ruchester, NY).

Identification of proHD-5 from small intestine. In order to compare the protein profiles, 5 mg of crude extract was developed by HPLC in the condition mentioned above. Samples of the collection fractions were determined by antimicrobial assay. The fraction with the strongest antimicrobial activity was applied for N-terminal protein sequencing. The whole single fraction was dried and loaded onto the NOVEX precast SDS–PAGE mini gels (Invitrogen, Carlsbad, CA), transferred to the PVDF membrane (Invitrogen, Carlsbad, CA), and stained with 0.1% Coomassie blue. The stained bands were then excited and applied to the protein sequencing (Biosum, Siga, Japan).

The molecular mass of the peptide included in the fraction was measured by matrix-assisted laser deionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Ibaragi, Japan). The expected mass of the proHD-5 was 8102.5 calculated as the peptide with three disulphide bonds in 6 cysteines.

Results

Recombinant peptide and the bactericidal activity

The purity of the peptides was assessed by AU–PAGE, in which the peptide migrates as each single band and the mobility depends on the size and the charge of the peptide. The HD-5 migrated faster than the proHD-5 since their numbers of amino acids are 32 and 75, respectively (Fig. 1A and B). The HD-5 was specifically reacted with the antiserum, checked by Western blotting (data not shown).

The antimicrobial activity, which was determined against *S. typhimurium*, demonstrated that both HD-5 and proHD-5 were bactericidal in 10 μg/ml concentration, which is estimated as a physiological concentration in the human intestinal lumen [10] (Fig. 1C).

Reduced recombinant defensins were digested by trypsin

The denatured peptides were chemically generated by reduction with DTT. AU–PAGE showed that the reduced

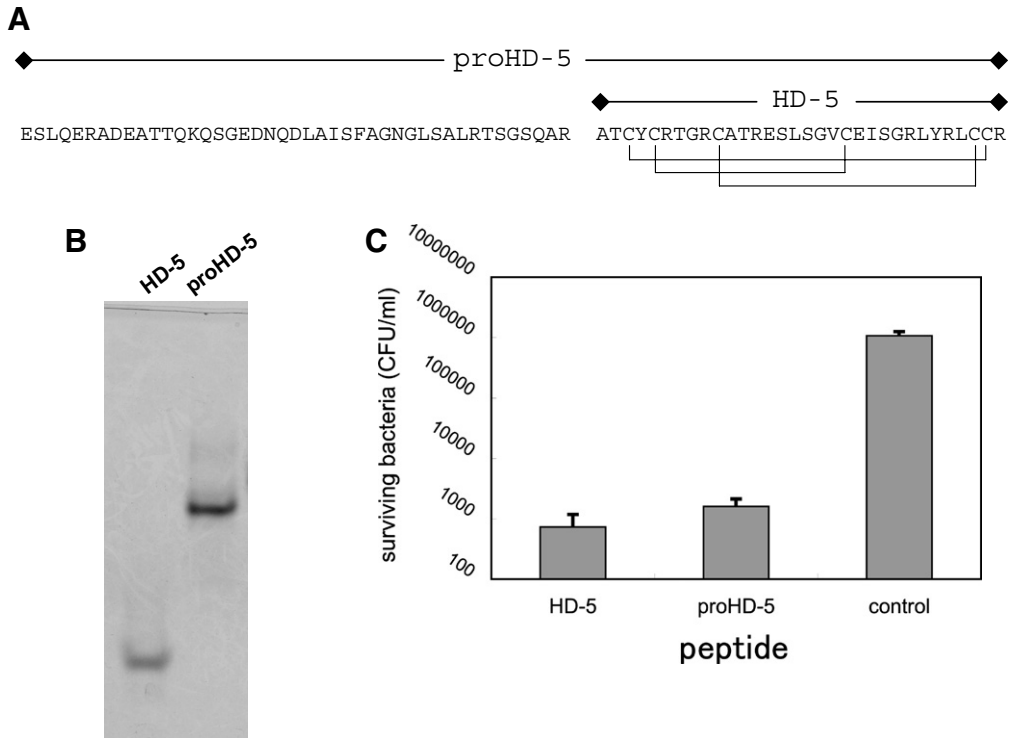

Fig. 1. Recombinant peptides of human enteric defensins. (A) Amino acid sequences of proHD-5 and HD-5. (B) Two micrograms of purified defensins were resolved on a 12.5% acrylamide AU–gel and stained with Coomassie blue. HD-5 moved faster than proHD-5 because mobility depends on size and charge of the peptide. (C) The bactericidal activity of each defensin was determined against *Salmonella typhimurium* PhoP-null. Each peptide (10 μg/ml in final conc.) was incubated with 10^6 CFU/ml of bacterial cells for 1 h and surviving bacteria were then counted. HD-5 and proHD-5 showed equivalent bactericidal activities.

form migrated significantly slower than the folded HD-5 and proHD-5, respectively (Fig. 2A and C). Since the reduced peptide is sensitive to trypsin treatment, the band was diminished in the gel. The folded proHD-5 was cleaved in order to form the mature HD-5 peptide which was resistant to the cleavage (Fig. 2C), confirming previous report [10]. The reduced peptides, which lost the bactericidal activities, demonstrated that the disulfide bonds confer not only the ability of stabilization of the molecule but also the physiological activity (Fig. 2B and D). The mass of the folded and the denatured proHD-5, confirmed with MALDI-TOF MS, was 8100.3 and 8105.9, respectively. The reduced peptide was 6 amino mass units longer than the folded peptide.

Defensin expressions in normal ileum

HD-5 from CD mucosa was sensitive to trypsin digestion

The expression of the peptides was determined by AU–PAGE Western blotting (Fig. 3). In order to detect the mature peptide from the crude extract, we applied trypsin cleavage. Mature HD-5 was not found in any of the materials without trypsin treatment, but it was detected as a fast migrating band in all of the samples from the normal small intestine after enzymatic digestion. In contrast, the mature HD-5 was diminished in 3 cases of the 6 CD patients (cases 3, 4, and 8), suggesting that the HD-5 that was derived from half of the CD patients was sensitive to trypsin and that the conformation of the mature active HD-5 was changed as the reduced recombinant defensins were digested by the trypsin.

Purification of denatured proHD-5 peptide from CD intestine

Small intestinal tissue was acid-extracted and 5 mg crude sample was developed by reversed phase-HPLC. The chromatograms showed no significant difference between the control and the CD patient (Fig. 4A). The defensin-rich fractions were determined by a bactericidal assay (Fig. 4B). The fraction demonstrating the strongest activity was then applied to the N-terminal protein sequence and ESLQERADEA…, which is identical to pro-peptide of HD-5, was obtained. A MALDI-TOF MS analysis showed 8102.0 amino mass units from the normal tissue (Fig. 4C, upper panel), identifying the pro-HD-5 including three disulphide bonds in the molecule. An HPLC fraction from CD case 3 was able to apply a mass spectrometry study and the 8107.6 amino mass units were observed (Fig. 4C, lower panel). The 6 amino mass units' increase, in comparison to the proHD-5 purified from the normal control, was identical to the reduced proHD-5. Therefore, the proHD-5 found in the CD intestine was expected to form an unfolded struc-

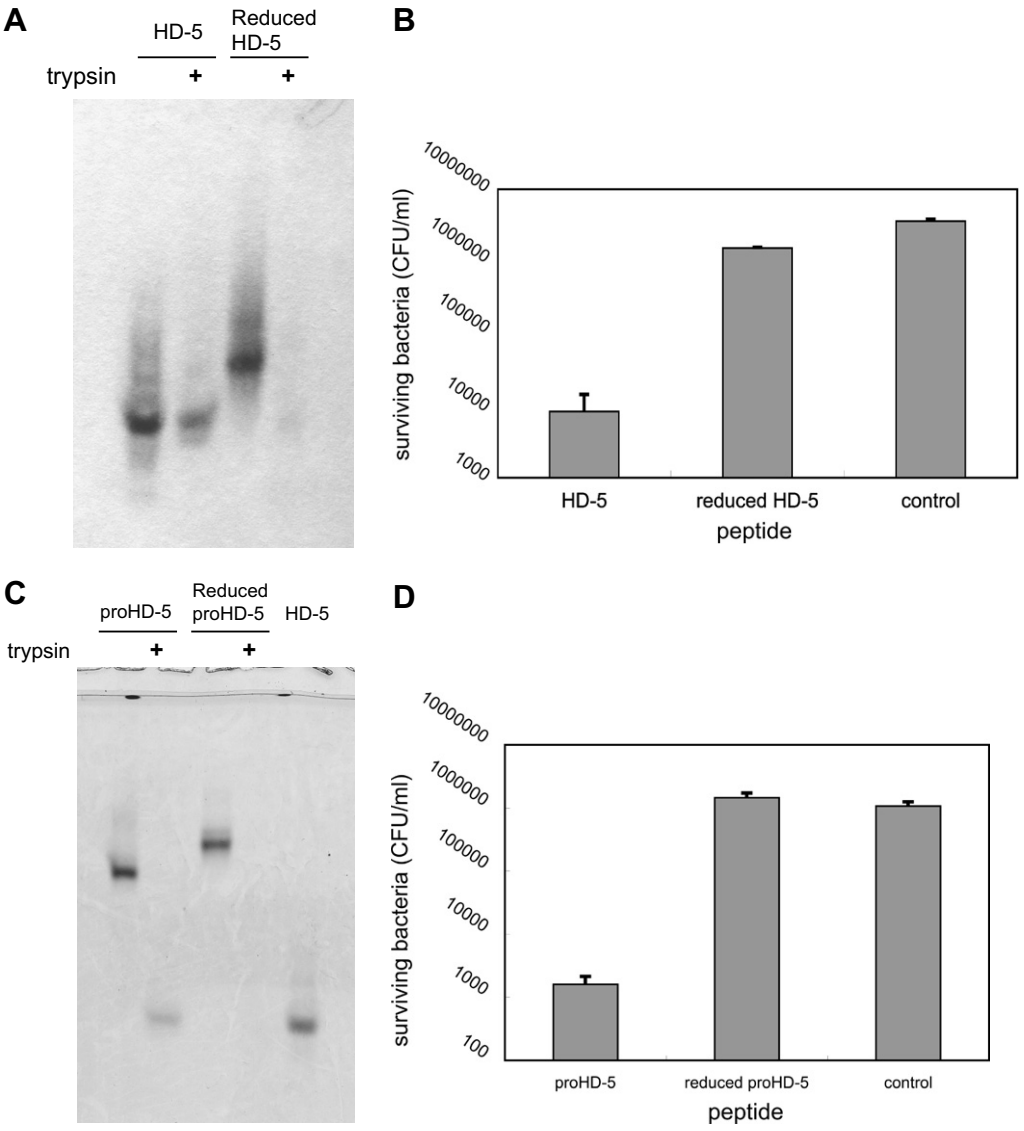

Fig. 2. Characterization of recombinant reduced HD-5 and proHD-5. (A,C) The recombinant HD-5 and proHD-5 peptides were reduced by DTT and purified by HPLC. Both forms were incubated with or without 0.5 μg of the trypsin overnight and loaded on AU–PAGE followed by Coomassie blue staining. The reduced forms moved slower than the folded form and were sensitive to the trypsin treatment. (B,D) Almost all of the bactericidal activity of HD-5 and proHD-5 (10 μg/ml in final conc.) were lost after the peptide was reduced by DTT.

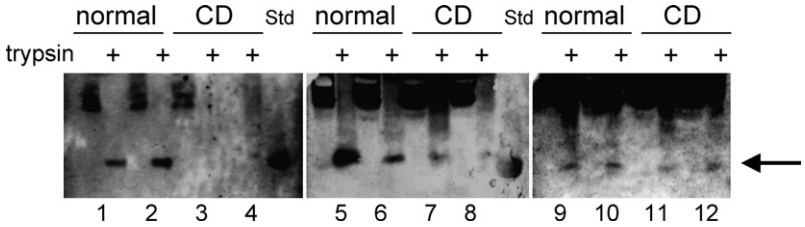

Fig. 3. Trypsin-sensitive HD-5 expression in CD small intestine. The samples (500 μg each lane) were loaded with AU–PAGE and transferred to the membrane for immunodetection. The mature HD-5 was detected as the fast migrating band (arrow). The trypsin sensitive HD-5 were observed in 3 out of 6 CD cases (cases 3, 4, and 8) and the expression of HD-5 was reduced in 3 other cases (cases 7, 11, and 12).

ture with no disulphide bridges. The reduced HD-5 was found in this study, demonstrating that the HD-5 peptide may be regulated by a post-translational modification in nature.

Discussion

The epithelial cells are the first barrier against luminal bacteria and respond to many bacterial antigens. The

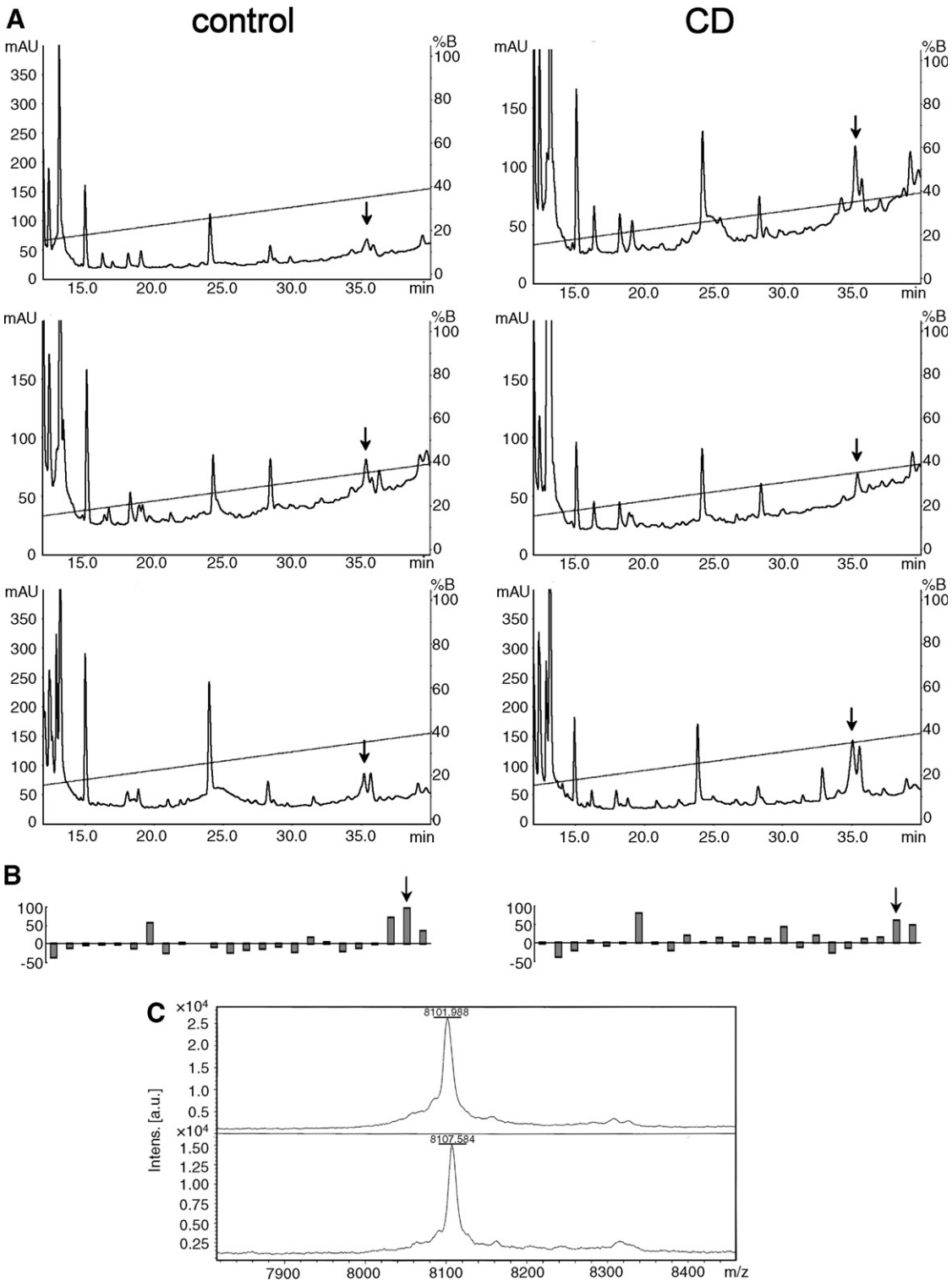

Fig. 4. Purification of native and reduced proHD-5 from the small intestine. (A) The intestinal crude extract was applied to the C18 column. The chromatogram patterns were compared between the control (left panel) and the CD patients (right panel). No significant differences were observed between these two groups. The arrows denoted the fractions containing proHD-5, determined with protein sequence. (B) Each fraction was applied to the bactericidal assay described in Materials and methods. The fractions of proHD-5 showed strong activity in both groups (arrow). Each bar presented the means of three experiments. (C) MALDI-TOF MS demonstrated that six amino mass units' increase in the CD patient (lower panel, case 3), presenting the disulphide bonds were reduced.

small intestinal Paneth cells, which are specialized columnar cells characterized by dense granules, are one of the major contributors to the innate immunity [2,21,22]. Two enteric defensins, HD-5 and HD-6, have been found in the Paneth cells [7,8]. The localization of HD-5 is determined by immunohistochemistry and its bactericidal

activity has already been demonstrated [9]. The Paneth cells are located at the bottom of the small intestinal crypt, and the granules are remarkably stained strongly in the serum.

One of the major roles of the Paneth cells is to act as a host defense against microbes secreting granules constituting an antimicrobial peptide mixture, determined with isolated crypts from the mouse small intestine [2]. The isolated crypts from the matrilysin knockout mouse lacks mature defensins and exhibits decreased antimicrobial activity. The *in vivo* study of the knockout mouse demonstrates the functional roles of defensins against orally challenged bacteria and the activation process [6]. Proteolytic activation is required for mouse intestinal defensins and this activation is completed in the Paneth cell granules before secretion. Mouse defensins are stored as mature forms in the intestinal tissue but human defensins are stored as pro-peptides and cleaved by a digestive enzyme, trypsin after the Paneth cell granules are secreted into the crypt rumen [10]. The bactericidal activity of the crude extract has been determined in recent papers [23]. In our study, proHD-5 was purified as the most bioactive molecule from low molecular proteins, though the mature HD-5 was not purified. The bactericidal activities of both the recombinant HD-5 and proHD-5 were also determined against *Salmonella* strains. It was concluded that HD-5 is stored as pro-peptides and already possesses bactericidal activity even without cleavage by trypsin, which is a cleavage enzyme of human enteric defensin after the Paneth cell granules are secreted into the luminal surface.

This activation process of the human enteric defensin is partially different from the mouse defensins that have been well studied in previous papers. The series of recombinant peptide studies has already demonstrated the relationship between the enzymatic activation and the characteristics of the disulfide bonds. Some of the experiments have focused on the roles of the disulfide bonds in the mouse defensins to demonstrate stability against enzymatic cleavage [11]. The disulfide bridges of human defensin contributed to stabilize the structure of the molecule and to keep the activity, as well as the mouse defensins. The human defensin was resistant to enzymatic digestion so that the trypsin treatment followed by AU–PAGE enabled us to detect the defensin as fast migrating bands. We coincidentally found that the HD-5 peptide diminished after the trypsin treatment in 3 cases out of 6 CD patients. We hypothesized that the process of disulfide bridge formation may be disturbed in the CD Paneth cells. The chromatograms of the CD intestine did not differ from the normal control. The peak of the proHD-5 was also conserved in all cases. The mass spectrum demonstrated the abnormal folding of the native proHD-5 that was purified from a CD patient. The protective roles of the disulfide bonds from the enzymatic degradation were also demonstrated using the recombinant peptides. We preliminarily found that the crypts that were isolated from the CD patients lacked bactericidal activity (data not shown). Taken together, the innate immune deficiency involved in CD may due to the abnormal activation process.

The intracellular synthesis of the disulfide bonds of the defensins remains unclear. For generating synthetic peptides with disulfide bonds, oxidative folding with reduced and oxidized glutathione has been successful [24], and the bacterial production of recombinant peptides has also been applied. The detailed mechanism of the modification, as well as mammalian production, will require further study. It is well known that protein folding is formed in the endoplasmic reticulum and that disulfide bonds are facilitated by the chaperones [25]. Therefore, stress induced reduction–oxidation unbalance in CD patients may cause a defensin reduction in the Paneth cells in the small intestine.

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