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Donkey milk lysozyme ameliorates dextran sulfate sodium-induced colitis by improving intestinal barrier function and gut microbiota composition

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ABSTRACT

Donkey milk lysozyme (DML) is a potential therapeutic agent for inflammatory bowel disease (IBD), while the mechanism remains obscure. Here, we reported that DML ameliorated weight loss, colon damage and mucosal inflammation in colitis mice. Additionally, DML increased the expression of occluding and zonula occludens-1, and reduced that of claudin-2, thus improving mechanical barrier function. It also inhibited the expression of tumour necrosis factor-α and interleukin-13, and reduced the myeloperoxidase level for strengthen of immune barrier function. DML increased gut microbiota composition diversity, promoting growth of probiotics and inhibiting pernicious bacteria, indicating that DML played an important role in microbial barrier function. The X-ray structure of DML was determined, which contained a core structure with conserved active site. The different conformation and residues of binding subsites may indicate diverse binding ability to substrates. These results suggested that oral administration of DML is a promising novel therapeutic for treatment of IBD.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic and recurring inflammatory condition of the gastrointestinal tract, affecting individuals throughout their life (Podolsky, 2002; Xavier & Podolsky, 2007), leading to tissue damage such as liver damage, arthritis, skin damage, myocardial disease, iris ciliary body inflammation and endocrine diseases. Notably, it has been established that longstanding colitis is a high risk factor for colorectal cancer (Lewis, Deren, & Lichtenstein, 1999; Pohl, Hombach, & Kruis, 2000). Although the aetiology of IBD remains unknown, it is widely believed that intestinal barrier function impairment, gut microbiota disturbance, and immune dysfunction caused by genetic and environmental factors play important roles in the pathogenesis of IBD (Frank et al., 2007; Ott et al., 2004; Papadakis & Targan, 1999). Numerous therapeutic strategies, including glucocorticoids, sulfasalazine, agents targeting proinflammatory pathways and immunosuppressive drugs, have been established for the treatment of IBD (Mao & Hu, 2016). However, because of their severe side effects and long-term toxicity (Atreya & Neurath, 2008), alternative effective therapeutic agents are urgently needed.

Donkey milk has become increasingly important in human nutrition, mainly because of its similar composition of proteins to human breast milk (Criscione et al., 2009). For this reason, donkey milk is considered a good alternative for infants with a severe protein allergy to bovine milk (Aspri, Economou, & Papademas, 2017). Donkey milk has been reported to have stronger microbial inhibitory activity and to result in lower levels of microbial contamination than the milk of any other species because of its high lysozyme levels (Labella et al., 2016). Notably, donkey milk exerted bactericidal activity against *Shigella. Dysenteriae* (Zhang, Zhao, Jiang, Dong, & Ren, 2008). The high lysozyme content of donkey milk directly inhibited tumour proliferation *in vitro* and showed a potent cytotoxicity to cancer cells via the induction of apoptosis, as well as indirectly killing tumours through the activation of lymphocytes and macrophages (Mao et al., 2009). Furthermore, belonging to the family of host defence proteins known as defensins, lysozyme modulated the aged immune system and the intestinal

Abbreviations: IBD, inflammatory bowel disease; DSS, dextran sulfate sodium; DML, donkey milk lysozyme; HEL, hen egg lysozyme; ZO-1, zonula occludens-1; MPO, myeloperoxidase; TJ, tight junction; TNF-α, tumour necrosis factor-α; IL, interleukin; PCoA, principal co-ordinates analysis

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mucosal immune response (Jirillo, Jirillo, & Magrone, 2010). Recent evidence has indicated that hen egg lysozyme (HEL) is a potent anti-inflammatory and immunomodulatory agent, which could attenuate dextran sulfate sodium (DSS)-induced inflammation and played an important role in the mucosal modulation of the immune response and restoring gut homeostasis (Lee et al., 2009). It is thus an attractive alternative to drug therapy in IBD (Lee et al., 2009). However, because of the allergenic effect, HEL has been replaced by donkey milk as an inhibitor of blowing caused by clostridia and coliforms in ewe cheese making (Cosentino, Paolino, Freschi, & Calluso, 2013). Therefore, the therapeutic function of donkey milk and lysozyme in IBD are widely considered to represent a significant breakthrough in the field of drug therapy in IBD research. As yet, it is not clear whether donkey milk or donkey milk lysozyme (DML) could alleviate the colitis, as well as the detailed mechanism.

DML is a c-type lysozyme that is 129 amino acids long and has 50% homology with human lysozyme (Godovac-Zimmermann, Conti, & Napolitano, 1988). It catalyses the hydrolysis of 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine residues in the peptidoglycan of bacterial cell walls, resulting in protection against bacterial infection (Labella et al., 2016). Lysozyme has a small compact globular structure very resistant to proteases and can also function in the digestive tract to reduce the incidence of gastrointestinal infections in infants (Vincenzetti et al., 2008). However, the catalytic mechanism of donkey milk-derived lysozyme remains unclear, and no three-dimensional structure has been obtained to date, restricting the functional research on DML.

Among the experimental models for IBD, DSS is commonly used to induce experimental colitis and colon cancer (Okayasu et al., 1990) characterized by weight loss, loose stools/diarrhoea, rectal bleeding, epithelial cell damage, and infiltration of granulocytes and mononuclear immune cells (Kawada, Arihiro, & Mizoguchi, 2007). In this study, by comparing donkey milk and cow milk we investigated the ability of donkey milk to reduce the symptoms and pathology of DSS-induced colitis, and confirmed the active component as lysozyme. We also evaluated the effect of DML supplementation on local protein expression of tight junction proteins (occludin, zonula occludens-1 (ZO-1), claudin-2), and pro-inflammatory and anti-inflammatory cytokines using a mouse model of experimental colitis. In addition, the gut microbiota was also analysed to determine the specific microbial clades associated with health, active disease and DML treatment intervention in a mouse model of colitis. To the best of our knowledge, we reported for the first time the crystal structure of DML at a resolution of 1.7 Å. Through analysis of its structure, this study provided a structural basis for understanding the conserved catalytic mechanism of DML. It also revealed a potential therapeutic agent for the treatment of IBD, and provided theoretical support for the development of functional donkey milk products and the extraction of functional components.

2. Methods

2.1. Donkey milk lysozyme isolation

Donkey milk was three-week to seven-week lactation milk provided by DEEJ Co. Ltd. (Shandong, China). Whey protein was obtained by centrifugation at 120,000*g* centrifugal for 60 min followed by dialysis for two-days (molecular weight 10 kDa) at 4 °C. Lysozyme was isolated by precipitating α-lactoalbumin (α-la) and β-lactoglobulin (β-lg) at pH5.5 and pH4.5 then lyophilized, and protein concentration was determined using the BCA method.

2.2. Animals and experimental design

Sixty six-week-old male Balb/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were housed five per cage in a controlled environment (12:12 h light-dark cycle, temperature 22 ± 1 °C) with free access to food and water. After one week acclimatization period, Mice were equally randomized into six groups: control, saline (oral administration of 0.85% NaCl for 21 days, the same below), la/lg (oral administration of 330 mg/kg BW of whey proteins containing α-la and β-lg without lysozyme), 5% DML (oral administration of 330 mg/kg BW of whey proteins containing 5% lysozyme and 95% α-la and β-lg), 20% DML (oral administration of 330 mg/kg BW of whey proteins containing 20% lysozyme and 80% α-la and β-lg) and 50% DML (oral administration of 330 mg/kg BW of whey proteins containing 50% lysozyme and 50% α-la and β-lg) groups. After 14 days of gavage treatment, the saline, la/lg, and 5%, 20% and 50% DML groups were treated with 3% DSS (MP Biomedical, Santa Ana, CA, USA) dissolved in drinking water to induce colitis in mice for 7 days. The mice with different treatment followed the same procedure mentioned before.

During the treatment, body weight, frequency and degree of diarrhoea and bloody stools were assessed every day. At the end of the experiment, mice were euthanized and the colons were carefully dissected. The length of colons was measured and recorded. Specimens of distal colon (approximately 1 cm) were fixed flat in 10% buffered formalin for histological analyses. The colon contents were collected and submerged in 1 ml RNA later (Qiagen, Hilden, Germany) at 4 °C for microflora analysis. The remaining tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until subsequent analysis.

2.3. Histological analysis

Colon samples were fixed flat in 10% buffered formalin then rinsed and embedded in paraffin. Five-μm-thick colon sections were stained with hematoxylin & eosin (H&E, Sigma, Steinheim, Germany) and representative photomicrographs were captured using the microscope at ×100 objectives.

2.4. Measurement of myeloperoxidase (MPO) activity

The ability of MPO to modulate the hydrogen peroxide level was used to measure MPO activity by using MPO Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The freshly excised colon tissue was rinsed and homogenized in tissue lysis buffer. Chromogenic agent 3,3-dimethoxy-4,4-diaminobiphenyl was added at 37 °C for 30 min before detection. The absorbance was then recorded at 460 nm. Finally, MPO activity was defined as the quantity of enzyme degrading 1 μmol/ml of peroxide at 37 °C, expressed in unit/g tissue.

2.5. Measurement of occludin, ZO-1 and claudin-2, TNF-α and IL-13 concentrations in colon tissue

For total protein extraction, colons were homogenized at 4 °C in a lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 20 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 5 mM EDTA, 0.2 mM Na3VO4, 2 μg/mL leupeptin, 2 μg/mL pepstatin A) with 1 mM PMSF then centrifuged at 12,000*g* and 4 °C for 30 min. The supernatant was collected and protein concentration determined using the BCA method. Occludin (DLdevelop, Wuxi, Jiangsu, China), Claudin-2 (DLdevelop, Wuxi, Jiangsu, China) and ZO-1 (Elabscience, Wuhan, China), TNF-α (eBioscience, California, USA) and IL-13 (DLdevelop, Wuxi, Jiangsu, China) concentrations were measured using ELISA Kits according to the manufacturer's instructions.

2.6. Analysis of microbiota in colon contents

Microbiota was measured by high-throughput sequencing. Bacterial DNA was extracted from colon contents samples. The V4-V5 regions of the bacterial 16S ribosomal RNA gene were amplified by PCR. Amplicons were extracted from 2% agarose gels and purified using the

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