S9A Serine Protease Engender Antigenic Gluten Catabolic Competence to the Human Gut Microbe

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Abstract The human gut microbiome has a significant role in host physiology; however its role in gluten catabolism is debatable. Present study explores the role of human gut microbes in gluten catabolism and a native human gut microbe *Cellulomonas* sp. HM71 was identified. SSU rDNA analysis has described human gut microbiome structure and also confirmed the permanent residency of *Cellulomonas* sp. HM71. Catabolic potential of *Cellulomonas* sp. HM71 to cleave antigenic gluten peptides indicates presence of candidate gene encoding biocatalytic machinery. Genome analysis has identified the presence of gene encoding S9A serine protease family—prolyl endopeptidase, with Ser591, Asp664 and His685 signature residues. *Cellulomonas* sp. HM71 prolyl endopeptidase activity was found optimal at pH 7.0 and 37 °C with a K_M of 35.53 μ mol and specifically cleaves at proline residue. Current study describes the gluten catabolism potential of *Cellulomonas* sp. HM71 depicting possible role of human gut microbes in gluten catabolism to confer resistance mechanisms for the onset of celiac diseases in populations with gluten diet.

Keywords Human gut microbes · Culture dependent techniques · SSU rDNA · Gluten protein · Celiac disease

Introduction

More than a billion microbial cells are residing all over the human body, which are higher in number compared to human cells [1–3]. Human microbiome is extremely diverse with spatial abundance across the body locations [1–4]. Amidst other body locations, human gut microbiome is highly dense and comprises of trillions of bacterial cells, approximately ten times more than the human cells [1, 3, 5]. Bacteroidetes and Firmicutes are dominant microbial groups within gut [2, 4], followed by Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria in lesser proportions [1–3, 5]. A variety of molecular studies have decoded the impact of the gut microbiota in cardiovascular diseases, irritable bowel syndrome, diabetes, and cancer [5–7]. A number of association studies link human nutrition and health with the metabolic capacity of gut microbiota [6–10]. However, insights into role of the human gut microbiome in gluten catabolism await further understanding.

Wheat gluten is one of the major constituent of the total dietary protein consumption. Gluten has high content of proline and glutamine residues in their structure, which are largely resistant to cleavage by the major human gastrointestinal (GI) digestive enzymes [11]. Partial cleavage of gluten generates immunogenic gluten peptides [11, 12]. These peptides activate CD⁺T cells in the lamina propria of HLA-DQ2 mediated immune system, followed by immunological inflammation of mucosa leading to gluten sensitivity and subsequent onset of celiac disease [11, 13, 14]. Recently, various oral cavity microbes and gut

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microbes were reported for their bio-catalytic role in gluten catabolism [15–19]. These studies emphasize on the role of human gut microbes in gluten catabolism and their possible role in ameliorating celiac disease [15–18]. Recent culture dependent and culture independent studies has decoded the functional role of a fraction of the human gut microbes (~ 10–20%) and indicates that majority of human gut microbiome still awaits functional elucidation. Present study was aimed to explore the functional role of human gut microbes in gluten catabolism. A native human gut microbe *Cellulomonas* sp. HM71 was identified for its gluten catabolic potential, illustrating possible role of human gut microbes in dietary gluten catabolism to avoid onset of celiac disease.

Materials and Methods

Sample Collection

Fecal sample was collected in a sterile container from a healthy individual (Age 29, Male, Blood Sugar 100–120 mg/dl, BP 120/80), with no past history of prolonged illness or antibiotic treatment. Fresh sample was used to culture microbes and isolate metagenomic DNA. Human ethical guidelines were followed strictly before engaging individual for current study. The study has been conducted after ethical clearance from the human ethical committee at Maharshi Dayanand University, Rohtak, Haryana, India.

Microbial Community Structure of Human Gut Microbiome

A 100 mg of fresh feces was used for extraction of metagenomic DNA [4]. The qualitative and quantitative analysis of the metagenomic DNA was performed with NanoQuant (Tecangrp. Ltd, Switzerland) and Qubit® dsDNA HS Assay Kit (Life technologies, USA). Metagenomic DNA isolated from healthy human stool sample was amplified for SSU rRNA gene [20]. An amplified product was sequenced with next generation sequencing using Roche 454 GS FLX+ system. Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 pipeline was implemented for data analysis. SSU rRNA gene sequences were quality filtered [20, 21].

Glutenase Activity in Stool Microbial Pellet

To confirm the role of human gut microbes in gluten catabolism, microbial pellet was purified from fresh human feces [4]. Bacterial cells were ruptured by ultra-sonication at 4 °C for 10 min with an output of 650 W (on/off pulse

of 9 s). The lysed cells were centrifuged at 13,000 rpm for 20 min at 4 °C to collect the supernatant, which was used for checking biocatalytic activity against the Z-Gly-Pro-pNA substrate [15].

Screening of Glutense Positive Microbe

Glutense active human gut microbes were isolated from fresh stool. A 50 mg of human feces were suspended in 1 ml phosphate buffer saline (PBS) pH 7.4. Suspension was centrifuged at 3000 rev min⁻¹. Debris free fecal suspension was serially diluted and plated on to glutense screening medium (Luria–Bertani broth (LB) supplemented with 1% gluten). Screening medium plates were incubated at 37 °C and observed for the presence of a hydrolytic halo surrounding the colony. Glutense active microbial culture was purified with the dilution culturing method and glutense activity of microbes was reassessed.

Phylogenetic Analysis of Glutense Positive Microbe

Genomic DNA was isolated from overnight grown culture of glutense positive microbe [22]. The SSU rRNA gene was amplified from the microbial genomic DNA to analyze its phylogeny [23]. Phylogenetic tree was constructed by using maximum likelihood method with help of MEGA-6 Package software version 6.06. The reliability of each branch was checked after performing bootstrap for 500 replicates [23].

Analysis for Gluten Catabolic Potential of *Cellulomonas* sp. HM71

Antigenic fraction of gluten was prepared from wheat gluten [24]. Gluten catabolic potential of *Cellulomonas* sp. HM71 was checked against synthetic substrate [15] and gluten antigenic fraction. Catabolism of antigenic gluten fraction was observed in a reaction mixture (2 ml) containing actively growing culture [$A_{600nm}(0.5)$], antigenic peptide (5 mg/ml) in PBS (pH 7.4). Reaction mixture was incubated at 37 °C and fractions were collected at different time intervals (0, 2, 4, 6 and 24 h). Fractions were heat inactivated and catabolism of antigenic peptide was observed with reverse-phase HPLC using a C-18 column [RP18 column (3.9 × 150 mm)] (Waters, USA) [16].

In Silico Characterization of *Cellulomonas* PEP Gene

Prolyl endopeptidase (PEP) gene was retrieved through genome survey of *Cellulomonas* sp and PEP gene sequence was characterized by using different open source bioinformatic tools [25].

Enzyme Activity and Substrate Specificity of *Cellulomonas* sp HM71 Prolylendopeptidase

Kinetic properties of *Cellulomonas* sp HM71 prolylendopeptidase were analyzed with Z-Gly-Pro-4-nitroanilide substrate [15]. All assays were carried out in the triplicate to calculate standard deviation. Substrate specificity study of *Cellulomonas* sp HM 71 was checked with Suc-Ala-Pro-pNA, Z-QQP-pNA, Z-YPQ-pNA (Helix Biosciences, India) and Z-Gly-Pro-4-nitroanilide (Sigma Aldrich) [15].

Results and Discussion

Microbial Community Structure of an Individual Gut Microbiome

Various attempts have been made to decipher human gut microbial community structure and found significant presence of *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Acidobacteria* [1–6, 26–28]. These microbes were characterized for their physiological functions, however a large portion of them still remains untapped. In the current study, an effort has been made to understand the functional role of resident gut microbes in gluten catabolism by decoding its native gut microbiome structure and identifying microbes with gluten catabolic potential. We obtained 46317 SSU rRNA gene sequences from gut metagenomic DNA. These sequences were curated for V1–V4 regions of the SSU rRNA gene and ambiguous, low quality (< Q30) and chimeric sequences were removed, resulting in a total of 8602 high quality reads for downstream analysis. All sequences were processed using QIIME 1.9.1 with de Novo clustering option, resulting in 696 OTUs. The average Shannon diversity index was 6.34. The even rate of identification of new OTUs, in reference to the number of SSU rRNA gene sequences was 12.35. Reads were assigned into operational taxonomic units (OTUs) using a closed reference OTU picking protocol in QIIME 1.9.1, *uclust* to search sequences against a subset of the Greengenes database, version (13_8) with a filtration parameter of 97% sequence identity. This resulted in 11 microbial phyla, inclusive of *Bacteroidetes* (88.7), *Firmicutes* (7.54%) and *Proteobacteria* (2.64%), followed by *Lentisphaerae* (0.21%), *Actinobacteria* (0.21%), *Gemmatimonadetes* (0.19%), *Acidobacteria* (0.07%), *Chloroflexi* (0.03%), *Verrucomicrobia* (0.01%), *Planctomycetes* (0.01%) and *Nitrospirae* (0.01%). Only 0.29% of the reads matched with unclassified/unknown microbial groups. Abundance of *Bacteroidetes*, *Firmicutes* and *Proteobacterial* microbial groups within human gut microbiome is in agreement with other human gut microbiome studies [29]. This corroborates that

most of the individuals share a common pool of microorganisms with inter-individual variability/individuality [30, 31]. This variability could be accounted by diet, physiology, geography and ethnicity [28].

Human gut microbes have efficient enzymatic machinery for catabolism of various dietary components like carbohydrates [32], polyphenols [33] and lipids [34]. However a limited information is available about the functional role of human gut microbes in gluten catabolism [16–19]. Accordingly, the human gut microbes were purified and checked for their gluten catabolic potential. Enzymatic assay with the microbial pellet lysate, hydrolyzed Z-Gly-Pro-pNA (synthetic enzymatic substrate for prolyl endopeptidase) and released 182 μ M free pNA. Similar enzymatic activity was observed with agar plate assay, where gluten (1%) was supplemented in agar medium. The results highlighted the possible role of human gut microbes in gluten metabolism.

Glutenase activity screening identified a highly active gluten catabolic microbial strain, HM71. The glutenase positive microbe HM71 was checked for its phylogeny using SSU rRNA gene analysis. SSU rRNA gene from glutenase positive HM71 was sequenced and assembled to generate a sequence of 1384 bp that shared a good homology (98–99.3%) with various *Cellulomonas* species like *Cellulomonas firmi* and *Cellulomonas biazotea*. Even similar phylogenetic affiliation was observed within Phylogenetic tree (Fig. 1a). Accordingly, human gut microbe HM71 was identified as *Cellulomonas* species strain HM71. Previous studies have also shown the presence of various *Cellulomonas* sp. from human feces [29] and characterized their cellulolytic machinery which was also found associated with *Cellulomonas* species HM71. A significant number of BLAST Hits of *Cellulomonas* species HM71 SSU rRNA gene sequences were observed within in-house SSU rRNA gene dataset, as well as in SSU rRNA gene dataset of Human Microbiome Project (HMP) (hmpdacc.org/) during abundance analysis (Fig. 1b). These results clearly demonstrate its nativity as a human gut microbe.

Gluten Catabolic Potential of *Cellulomonas* sp. HM71

Time series analysis of antigenic gluten catabolism with *Cellulomonas* sp. HM71 has indicated a significant degradation of antigenic peptide (> 95% %) within 4 h of incubation (Fig. 2). Catabolism of antigenic gluten fraction confirms gluten catabolic potential of *Cellulomonas* sp. HM71. *Cellulomonas* sp. HM71 also showed a remarkable biocatalytic activity against Z-GP-pNA, SAP-pNA and Z-QQP-pNA synthetic substrates for glutenase [35], while a poor activity against Z-YPQ-

Fig. 1 SSU rRNA gene sequence analysis. Phylogenetic analysis of glutenase positive human gut microbiome HM71 (a), abundance of *Cellulomonas* sp. HM71 SSU rRNA gene sequence homologs within

NIH- HMP stool SSU rRNA gene sequence dataset and in house human fecal SSU rRNA gene dataset (b)

Fig. 2 *Cellulomonas* sp. HM71 hydrolytic activity against gluten antigenic fraction. HPLC chromatogram and peak area were checked after incubating gluten antigenic fraction with *Cellulomonas* sp. HM71 for different time period (0, 2, 4, 6 and 24 h)

pNA (Fig. 3). These results indicate its proline specific biocatalytic activity with potential to hydrolyze the immunogenic gliadins that could lead to the onset of celiac disease [12, 29, 30]. *Cellulomonas* sp. HM71 possibly has hosts bioactive S9A serine proteases.

Presence of gluten catabolic potential in a human gut microbe indicates possible role of human gut microbes to confer resistance mechanisms for declining onset of celiac diseases in populations with gluten diet.

Fig. 3 Substrate specificity of *Cellulomonas* sp. HM71 prolyl endopeptidase

In Silico Characterization of *Cellulomonas* sp. HM71 PEP Gene

Many bacterial and fungal species are reported to harbor gene for prolyl endopeptidase [3, 14]. A genome survey of *Cellulomonas* species has identified *PEP* genes within *Cellulomonas* microbial clade. *PEP* gene encodes a protein of 728 amino acids and molecular weight of 78kD. The encoded protein belongs to serine protease subfamily S9A within the esterase super family (Supplementary Fig. 1). The *PEP* protein was checked for various physiochemical

characteristics (Table 1). These analyses indicated its aqueous solubility and stability with cytosolic topology. Similar characteristics were observed for *Myxococcus xanthus* prolyl endopeptidase [19]. Multiple sequence alignment of *Cellulomonas* PEP with other characterized PEP protein has indicated the presence of three active residue Ser591, Asp664, His685, as described in other studies [35].

Kinetic Properties of *Cellulomonas* sp HM71 Prolyl endopeptidase

Prolyl-endopeptidase of *Cellulomonas* sp. HM71 showed its biocatalytic activity in the pH range of 6.0–9.0 (Fig. 4), with an optimum pH of 7.0 at 37 °C in 50 mM phosphate buffer, with unit activity of 3.22 μM min⁻¹ with K_M and V_{max} of 35.53 μmol and 0.362 μmol min⁻¹ respectively. We further investigated whether the function of microbial prolyl endopeptidase was pH dependent. The enzyme pH activity profile was matched with gently acidic environment of the upper small intestine (pH 6–8) that provides the clue about the metabolic site of gluten in human intestine. The cleavage site specificity of the PEP enzyme is at SAP↓, GGP↓, TP↓G and GP↓. The cleavage activities are highly active against dipeptide GP↓ and tripeptide GGP↓ at proline residue site. On the basis of its active sites and cleavage properties, it was confirmed that our microbial enzyme has sufficient potential for degrading the gluten. Findings in present study have potential role of human gut microbes in management of celiac disease. Till date, a

Table 1 Physiochemical characterization of PEP sequence

Characteristics	PEP
Protein super family	Esterase lipase
Protein family	Serine 9A
EC number	EC 3.4.21.26
Amino acids	728
Molecular weight	78,393.1 Da
Theoretical pI	5.07
Ext. coefficient (all pairs of Cys residues form cystines)	138,895
Ext. coefficient (all Cys residues are reduced)	138,770
Estimated half-life	30 h (mammalian reticulocytes, in vitro) > 20 h (yeast, in vivo) > 10 h (<i>Escherichia coli</i> , in vivo)
Instability index	35.11
Subcellular localization	Cytosolic
Hydrophobicity	No
N-Glycosylation site	No sites predicted
Prolyl endopeptidase serine family	509–727
Active site residues	Ser591, Asp664, His685 (Position as per gene encoded sequence)
Conserved domains	Prolyl oligopeptidase
Signal peptide	No

Fig. 4 Enzymatic analysis of *Cellulomonas* sp. HM71 prolylen-dopeptidase at different pH

limited information is available about the existence of gluten-degrading microorganisms in the human gut [16–19] and absorption/detoxification of gluten in human gut is still a mystery. The current study has identified a human gut microbe with a potential to detoxify the antigenic gluten peptides. The present study highlights the role of human gut microbes in gluten catabolism and identifies faecal bacteria that secrete gluten degrading enzyme. These bacteria and its secreted enzyme could lead to novel and effective strategies to detoxify immunogenic gluten peptides prior to reaching the proximal small intestine.

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Compliance with Ethical Standards

Ethical Statement The current study describes the functional role of human gut microbe isolated from human faeces of a human participant. Accordingly, human ethical guidelines were followed strictly and an ethical clearance was sought from human ethical committee of Maharishi Dayanand University, Rohtak, Haryana, India for conducting the present study. Along with this, this article does not contain any studies with animals performed by any of the authors.

Conflict of interest All authors declare that they have no conflict of interest.

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