Journal of Microbiological Methods 123 (2016) 31–38

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Assessing the impact of long term frozen storage of faecal samples on protein concentration and protease activity

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ARTICLE INFO

Article history:
Received 12 November 2015
Received in revised form 29 January 2016
Accepted 1 February 2016
Available online 4 February 2016

Keywords:
Gut
Microbiota
Proteases
Proteins
Long-term storage
Enzyme activity

ABSTRACT

Background: The proteome is the second axis of the microbiome:host interactome and proteases are a significant aspect in this interaction. They interact with a large variety of host proteins and structures and in many situations are implicated in pathogenesis. Furthermore faecal samples are commonly collected and stored frozen so they can be analysed at a later date. So we were interested to know whether long term storage affected the integrity of proteases and total protein and whether historical native faecal samples were still a viable option for answering research questions around the functional proteome.

Methods: Faecal samples were collected from 3 healthy volunteers (3 biological replicates) and processed in order to be stored at both −20 °C and −80 °C and in a variety of storage buffers. Protein extraction, protein content and protease activity were assessed at the time of collection, after 24 h, 1 week, 1 month, 3 months 6 months and finally 1 year.

Results: Beadbeating impacted the quantity of protein extracted, while sodium azide did not impact protease assays. Long term storage of extracted proteins showed that both total protein and protease activity were affected when they were stored as extracted protein. Intact faecal samples were shown to maintain both protein levels and protease activity regardless of time and temperature.

Conclusions: Beadbeating increases the protein and protease activity when extracting from a faecal sample, however, the extracted protein is not stable and activity is lost, even with a suitable storage buffer. The most robust solution is to store the proteins in an intact frozen native faecal matrix and extract at the time of assay or analysis, this approach was shown to be suitable for samples in which, there are low levels of protease activity and which had been frozen for a year.

1. Introduction

The human gut harbours microorganisms whose collective gene content outnumbers the host's own exome by at least 100 to 1 (Gill et al., 2006). While many studies have tried to understand interactions between bacteria and the host via a pathogen:host interactome model, we are currently exploring a commensal:host model and where disease appears, an amensal:host model. In the last two models the microbiota communicates with the host either by the metabonome or metaproteome. In order to make sense of the new form of dialogue many groups are implementing 'omic' based technologies, in particular, metagenomics, metabonomics and metaproteomics to elucidate the microbial functions responsible for the interactions, and the effectors which are derived from these functions. One such aspect of this molecular dialogue is the enzymes made by bacteria, which interact with host components to exert an influence on host functions, physiology and anatomy.

Faecal samples are often used as a surrogate for the gut microbiota (Eckburg et al., 2005) since they are considered an easy, safe and reputable proxy for describing both intra- and inter-personal differences in the gut microbiota composition and function (Peterson et al., 2008). In metagenomic studies DNA isolation is one of the most important steps and many parameters have been investigated which can affect both the quantity and quality of the nucleic acids extracted (Bertrand et al., 2005, Cowan et al., 2005, Ekkers et al., 2012, Purohit and Singh, 2009, Salonen et al., 2010). Long term storage of DNA extracted from faecal samples as well as other environments has also been extensively studied (McOrist et al., 2002, Salonen et al., 2010, Yuan et al., 2012). However, as the frequency of metaproteomic studies increases, so does the necessity to develop optimal protein extraction methods (Kolmeder et al., 2012, Lacerda and Reardon, 2009, Macfarlane et al., 1986, Tanca et al., 2014a,b). However, proteins can exist in many different biological forms, even if isolated from the same environment and so developing a

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http://dx.doi.org/10.1016/j.mimet.2016.02.001

generic extraction method for proteins is particularly difficult (Wilmes and Bond, 2006). Furthermore, protein samples for metaproteomics often do not need to maintain their activity, whereas clearly in activity-based studies or functional proteomics, this is of paramount importance. Therefore there is a clear need to understand the impact of storage regimes on the enzyme activity as well as the quantity of proteins in a faecal sample.

Protein degradation by microorganisms in the human gut has been linked with colon cancer (Hughes et al., 2000) and specific bacterial proteases have been shown to influence invasiveness of colon tumour cells (Flintoft, 2003). Microbial proteases represent approximately 5% of the genomes of infectious organisms (Shen and Chou, 2009) and approximately 2% of the genomes of commensals (Rawlings et al., 2014). Protease activity has been studied by direct methods; Macfarlane and colleagues have given us valuable insights into the functionality of the healthy gut (Macfarlane et al., 1986) and to some extent, the unhealthy gut as well (Steck et al., 2012). Most studies on proteases and how they may be impacting the host are carried out on pure cultures of specifically chosen microorganisms which may not actually be numerically dominant in the gut (Steck et al., 2011, Wu et al., 1998, 2007). While these studies are incredibly insightful, and demonstrate how significant microbial proteases and their interactions with host cells are, it is also useful to study enzymatic functions as a separate entity i.e. proteases as they are found in a particular environment at any one time point. Stool samples offer this non-invasive insight into proteolytic activity of the human gut microbiota as it may be occurring in situ.

Thus the main aim of this work was to assess the parameters that may introduce variability upon storage of faeces for protein and protease studies. In this study, we tested a variety of variables which we hypothesise might influence protein stability and thus protease activity, these included, buffer composition, time, temperature, extraction method and preservatives.

2. Materials and methods

2.1. Buffer composition and preparation

All buffers were made up in molecular grade water (18 MΩ) and where possible steam sterilized at 121 °C for 15 min, where heat labile ingredients were present; buffers were filter sterilised (0.2 μm). Two buffers were chosen for futher assessment, and the composition for each one is shown in Table 1. All reagents used were available from Sigma (Poole, UK) unless otherwise stated.

2.2. Collection of faecal samples

Assays to detect and quantify enzymatic activities were developed and optimized with faecal specimens from laboratory volunteers (n = 3). Institutional ethical approval was obtained prior to the study and was granted by Cardiff School of Biosciences, Cardiff University (Cardiff University's Research Ethics Committee). Participants gave written consent following retrieval of a participant information sheet detailing all aspects of the study and for what their samples would be used.

Faecal samples were collected from healthy male participants from Cardiff University. All samples were processed and stored following correct protocol as determined by the Human Tissue Act (HTA) 2004 and samples were all anonymised. Samples were collected using disposable trays; participants were advised to ensure that no urine was collected in the tray alongside the faecal sample. Trays were deposited anonymously in a fridge at 4 °C until DNA extraction (the same day). The remaining sample was thoroughly mixed and divided into 1 g samples in sterile universal tubes and immediately frozen at −20 °C and −80 °C.

Table 1
Buffer composition.

Buffer	Buffer composition
1	PBS, 0.05% w/v NaN_3
2	PBS, 10% v/v glycerol, 0.05% w/v NaN_3

2.3. Protein concentration measurements and protease assays

Protein concentrations were assessed using the bicinchoninic acid assay according to the manufacturer's instructions (PIERCE, Rockford, IL, USA). General protease activity was determined by measuring the release of acid-soluble substance from azocasein (Sigma-Aldrich) over a period of 3 h after precipitation. Azocasein (5 mg/ml) was prepared in 50 mM Tris–HCL, pH 8. In order to assess protease activity in total protein samples (100 μl at 1 mg/ml protein (vide infra)) were added to the azocasein solution (100 μl). The mixture was incubated at 37 °C and the reaction was terminated by the addition of 400 μl of 10% (w/v) trichloroacetic acid (TCA). Protein was precipitated by centrifugation at 12,000 × *g* for 5 min and the resulting supernatant was transferred to a clean tube containing 700 μl 525 mM NaOH. The absorbance was measured using a spectrophotometer at 442 nm. Each reaction was carried out in triplicate. Negative controls were prepared by setting up a reaction and immediately terminating the reaction with TCA. The resulting precipitate was taken as a negative control. To minimise background interference, a further negative control was set up with just water. Proteinase K (Sigma Aldrich) was used as a positive control at a concentration of 2 μg/ml.

2.4. Design, conduct and analysis of storage media for evaluation of protein yield and protease activity over time

The experimental process is shown in supplemental Figs. S1 and S2. To expand on this, fresh material from one faecal sample (collected from 3 healthy volunteers in total) was collected and divided into 4 subsamples (1 g). One sample for each buffer and each sample would be used for total protein extraction (i.e. with bead beating) and for extracellular protein only analysis (no bead beating) each containing 1 g faecal material. Each sample was allocated to a buffer (1, or 2) and this buffer was added to the faecal sample to prepare a 10% w/v faecal slurry which was homogenised by mixing on a Vortex Genie 2™ until no clumps remained. To prepare the crude total protein extract the faecal slurry was divided into 2 ml RNAse and DNase free lysing matrix E tubes (MP Biomedicals) containing 1.4 mm ceramic spheres, 0.1 mm silica spheres and one 4 mm glass sphere. Samples were kept on ice throughout. The samples were subject to bead beating using a FastPrep-24 bead beater (MP Biomedicals) at a speed of 6.0 m/s for 30 s with a period of 5 min on ice between each beating. To determine the optimal number of bead beating steps this process was repeated up to 6 times. The bead beating step was repeated further 2 times for optimal recovery of intracellular protein. Samples were subject to centrifugation at 20,000 × g for 30 min at 4 °C and the supernatant from this step was filtered through a 100k Amicon Ultra centrifugal filter tubes (Millipore, Darmstadt, Germany) according to the manufacturer's instructions to allow proteases through. For the extracellular only samples, this centrifugation step was conducted immediately instead of the bead beating step. Supernatant after filtration was transferred to new sterile tubes and taken as the crude protein extract. Sodium azide (NaN_3) was added aseptically to each sample to a final concentration of 0.05% w/v. Samples were divided into 1 ml aliquots and stored at −20 °C. Neat samples, 10-fold and 100-fold dilution were used to estimate protein concentration using the bicinchoninic acid assay (BCA) method according to the manufacturer's instructions (PIERCE, Rockford, IL, USA) and samples were normalised to 1 mg/ml protein using the appropriate buffer as a diluent to conduct subsequent protease activity estimates. Azo-casein assay was performed as described above. The protein concentration measurements and protease activity estimates were