



From bedside to bench: Evaluating the effect of different storage and extraction techniques on protease activity analysis in inflammatory bowel disease patients

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ABSTRACT

Proteolytic enzymes (proteases) are integral to multiple physiological processes, and increased protease activity is observed in inflammatory bowel disease (IBD) and other gastrointestinal diseases. Therefore, protease activity has been explored for potential diagnostic biomarkers and therapeutic targets. Faecal samples are commonly used to measure protease activities associated with gastrointestinal conditions as they allow non-invasive access to the diseased environment. However, faecal sample storage and protein extraction methods are not standardised for the measurement of faecal protease (FP) activity. In addition to being of scientific importance, the evaluation of the viability of FP activity as a potential biomarker for IBD management is also of clinical interest. In this study, the effect of different sample storage conditions and extraction techniques on FP activity were measured, on samples from IBD patients, using fluorescent substrates highly selective for serine proteases. FP activity was found to be stable for up to 72 hours at both 4°C and room temperature. Activity was found to decline more rapidly when samples were stored in extraction buffer rather than as crude samples. Regarding extraction techniques, FP activity was highest when extracted in Tris based buffers at pH 7.5–8; bead-beating of samples during extraction was found to have no significant effect on activity; and processing the extracted sample on ice was shown to minimise loss of FP activity. These results provide a framework for standardised evaluation of FP activity and suggest that protease activities have the potential to be a viable future biomarker.

1. Introduction

Proteases constitute around 2 % of the human genome and are integral to multiple physiological processes including immunity, cell death, inflammation and coagulation [1]. Protease activity has been shown to be significantly increased in inflammatory bowel disease (IBD), as well as other gastrointestinal diseases, and there is increasing evidence that these enzymes play an important role in intestinal inflammation [2–7]. The ability to accurately measure and characterise protease activity is therefore of great importance in understanding IBD aetiology. In addition, protease activity has previously been considered as a target for treatment of gastrointestinal disease and may also represent a source of novel diagnostic biomarkers [1,8].

Proteins in the gut lumen may be detected in faeces and can provide a

non-invasive window into the diseased environment. Faecal samples are, therefore, commonly collected and used to analyse proteolytic activity in the human gastrointestinal tract [2]. However, faecal sample collection methods, storage and protein extraction protocols are not standardised for this process which means practice is likely to vary substantially. In particular, the storage and handling of faecal samples prior to analysis, from both healthy controls and patients with gastrointestinal disease, is often not described in detail. Understanding the effect that different stool storage conditions and protein extraction protocols have on faecal protease (FP) activity analysis will allow for standardised storage and processing methods, ensuring accurate and reproducible results when studying protease activity, and guiding the feasibility of developing real-world diagnostic tests. The most widely used faecal test for both the diagnosis and monitoring of IBD is faecal

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calprotectin which, whilst sensitive, has been shown to lack specificity in certain patient groups, necessitating the continued investigation of alternatives [9]. It is, however, stable at room temperature for long periods, making it well-suited for use in a wide variety of different clinical settings [10]. Any alternative diagnostic tests would need to meet similar levels of practicality for use in settings where immediate sample processing or deep freezing is not possible.

Existing literature suggests that FP activity is stable once samples are stored at either -20°C or -80°C , as crude faecal samples [11]. There are, however, limited data on the effect of time and conditions prior to freezing on protease activity although some studies have investigated the effect of pre-freezing conditions on other gut enzyme activities. Flores and colleagues investigated the microbial enzymes β -glucuronidase and β -glucosidase and suggested that longer times at room temperature prior to freezing resulted in reduced enzyme activity [12]. In contrast, Dumoulin and colleagues assessed leukocyte esterase activity demonstrating that activity was stable when faecal samples were kept at both room temperature and 4°C for up to 24 hours [13]. In addition, there are data looking at the effects of different sample processing and storage conditions on protein stability more generally, but with limited focus on the effect on measurable enzyme activity [14,15]. Regarding the effect of different extraction methods on FP activity, Morris and Marchesi examined the effect of bead beating, finding that its use increased protease activity measured using an azocasein based colourimetric method [11]. There are, however, few other studies assessing other methods of faecal protein extraction on enzyme activity.

In this study we investigated the effects of different storage conditions on serine-like protease activity in faecal samples from patients with active IBD. We focused on serine proteases as their activity has been shown to be increased in patients with active IBD, and the majority of human serine proteases are secreted making them ideal faecal biomarkers [2]. The effect of buffer type, bead-beating during extraction and sample handling temperature were also assessed. These results will help inform the development of standardised protocols for the analysis of FP activity, whilst confirming that activity is stable for long enough in stool samples to be a valid clinical assay.

2. Methods

2.1. Sample collection

All patients provided informed consent prior to providing samples

(IRAS ID 243310). In total 18 patients with active IBD were recruited. Active IBD was defined by a faecal calprotectin of $> 150\ \mu\text{g/g}$ or a Simple Clinical Colitis Activity Index (SCCAI) > 5 in the case of patients with Ulcerative Colitis and a Harvey-Bradshaw Index (HBI) > 4 for Crohn's disease [16,17]. For the evaluation of faecal sample handling and storage prior to freezing at -80°C , hospital inpatients with active IBD were recruited. Hospital inpatients were used so that faecal samples could be processed quickly once provided. Samples were provided in a faeces container (FECOTAINER®, AT Medical BV, The Netherlands) and aliquoted into nine 1.5 ml microcentrifuge tubes (Protein LoBind Tubes – Eppendorf; Hamberg, Germany). One sample was transferred to -80°C within one hour and the remaining samples were either stored at room temperature ($20\text{--}25^{\circ}\text{C}$) or 4°C , with further samples being transferred to -80°C at 6 h, 24 h, 48 h and 72 h (Fig. 1). For all other experiments, samples were collected from patients with active IBD using the same method with samples aliquoted and transferred to storage at -80°C within 6 h of defaecation.

2.2. Faecal supernatant sample preparation

To extract protein from faecal samples, ice-cold faecal protein (FP) extraction buffer was added at a ratio of 100 mg faeces/1 ml of buffer to samples in microcentrifuge tubes (Protein LoBind Tubes). Extraction buffer comprised 50 mM Tris and 150 mM NaCl at a pH of 7.5. This composition was chosen following a previous experiment investigating the effect of varying extraction buffer on protease activity (detailed in [Supplementary Material](#)). Samples were incubated on ice for 30 min, being vortexed every 10 min, before centrifugation for 20 min at 1850 g at 4°C . The supernatant was added to $0.22\ \mu\text{m}$ centrifuge tube filters (Costar Spin-X Centrifuge Tube Filters - 8161; Corning, USA) and centrifuged for 30 min at 15,800 g at 4°C and the flow through was collected for analysis.

2.3. Bead beating

Faecal samples from IBD patients were combined with extraction buffer and vortexed following the same methods documented in [section 2.2](#). Two samples were processed from each patient, one being allocated to undergo bead-beating during the extraction process, and one following the previously described method. For the sample allocated to bead-beating, prior to centrifugation at 1850 g, 0.1 mm glass beads (13118; QIAGEN, Manchester, UK) were added and three 20 second

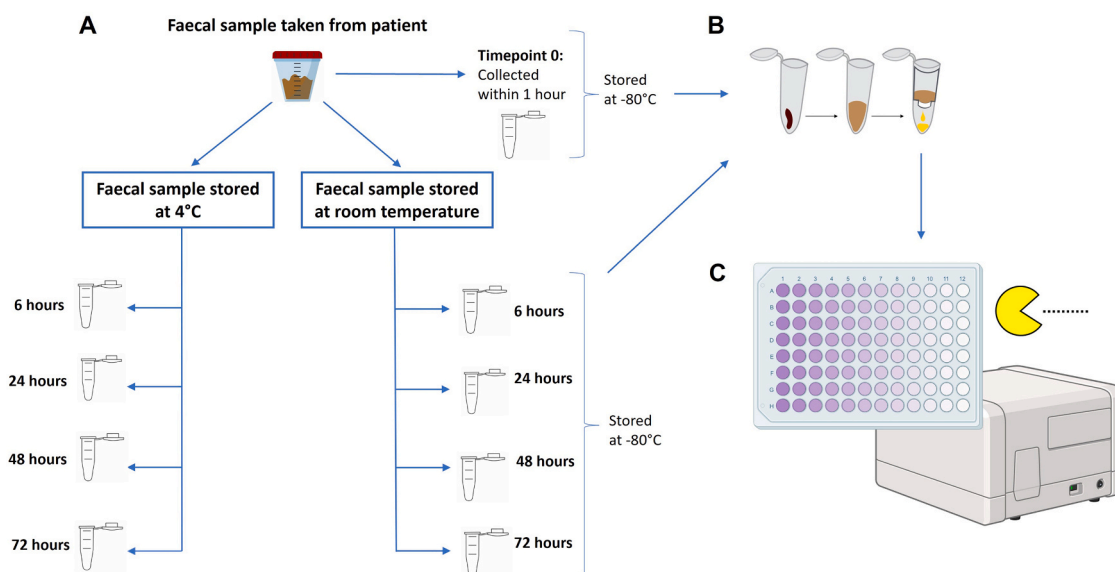


Fig. 1. Flowchart showing varying storage conditions of faecal samples prior to freezing at -80°C .

cycles of bead-beating were performed at 6000 rpm using a Precellys Evolution Touch Homogeniser (Bertin Technologies, France). Samples were kept on ice between cycles to avoid heating. Samples were then centrifuged at 1850 g and filtered before protease activity was assessed.

2.4. BCA assay for protein concentration

Total protein concentration was determined using the Pierce™ BCA (Bicinchoninic acid assay) Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) per the manufacturer's instructions and samples were normalised to a concentration of 1 µg/µl.

2.5. Protease activity assays

Protease activity was assessed using fluorogenic substrates. N-methylsuccinyl-Ala-Ala-Pro-Val-7- amino-4-methylcoumarin (M9771; Merck (Sigma-Aldrich), Darmstadt, Germany) was used at a 25 µM final concentration to measure elastase-like activity. N-p-Tosyl-Gly-Pro-Arg 7-amido-4-methylcoumarin hydrochloride (T0273; Merck (Sigma-Aldrich)) at a 100 µM final concentration was used to measure trypsin like activity and Chymotrypsin Substrate II (Suc-Ala-Ala-Pro-Phe-amino-4-methylcoumarin- 230914; Merck (CALBIOCHEM), Darmstadt, Germany) at a 100 µM concentration to measure chymotrypsin like activity. 50 µl of faecal supernatant was combined with 50 µl of each substrate in a black 96-well flat bottom plate (611F96BK; ThermoFisher Scientific, Ashford, UK) in technical duplicate. All substrates were made at double the desired concentration before being added to the faecal supernatant. Substrates were diluted in protein extraction buffer (50 mM Tris and 150 mM NaCl, pH of 7.5) to reach this concentration. Substrate hydrolysis was monitored by the cleavage of amido-4-methylcoumarin (AMC) from each substrate at an excitation of 360 nm and emission of 460 nm using a fluorescence microplate reader (FLUOstar Omega, BMG Labtech Ortenberg, Germany) in top reading mode. Readings were taken for a total of 60 min with intervals ranging between 13 and 30 sec.

2.6. Statistical analysis

Using GraphPad Prism (v9.5.1) the mean kinetic measurement from technical duplicates of each individual sample were plotted against time elapsed to produce a curve reflecting enzyme activity. A simple linear regression fit on the linear portion of each curve, corresponding to enzyme initial velocity, was performed to calculate the substrate rate of cleavage, expressed in change of relative fluorescent units per second per milligram of protein (rfu/s/mg). The linear portion of the curve was selected by visually inspecting the graph and ensuring the R² value of the simple linear regression performed on the segment was greater than 0.95. A repeated measures ANOVA with Tukey's multiple comparisons test was used to assess for any significant differences between the rates of cleavage of samples stored at multiple different conditions. A Wilcoxon test was used to compare the difference in rate of cleavage between samples exposed to bead-beating and no bead-beating, between samples stored as extracted faecal supernatant to crude faecal samples and between samples processed at room temperature compared to on ice. A Pearson's correlation coefficient was used to assess differences between replicate values.

3. Results and discussion

Several studies have suggested the potential use of protease activity as faecal biomarkers for IBD [3,5]. However, very little has been done to determine the clinical validity of protease activities for this purpose, specifically whether protease activities are stable for long enough to make any clinical assay viable. Here, the impact of different sample storage conditions and protein extractions methods on FP activity were assessed to attempt to answer this question and inform future collection

and storage protocols.

3.1. Stability of protease activity in faecal samples

3.1.1. Protease activity is stable up to 72 hours when stored as crude faecal samples

To analyse the stability of protease activity in crude faecal samples, stored for varying lengths of time and in different conditions prior to freezing, samples were collected fresh from three IBD patients and stored in the multiple conditions shown in Fig. 1. In total nine samples were analysed per patient across the difference storage conditions. Samples were processed and serine-like protease activity measured using fluorescent substrate assays. To enable accurate comparisons across samples, substrate cleavage rates were determined from the initial linear portion of the reaction curve, as this region is least affected by substrate depletion, product inhibition, or enzyme instability and most accurately reflects the enzyme's catalytic activity. Across the nine timepoints there was no significant change in trypsin-like, chymotrypsin-like or elastase-like activity seen for samples stored at either room temperature or 4°C for up to 72 hours (Chymotrypsin $p = 0.433$, Elastase $p = 0.372$, Trypsin $p = 0.480$; Fig. 2 A-C). Results were consistent between technical replicates showing a strong correlation ($r = 0.996$) validating the reliability of the fluorescent substrate assays (Fig. S3). In one of the three patients there was a large numerical decline in chymotrypsin-like activity between the baseline sample and the samples kept at room temperature and 4°C for 6 h prior to freezing (rate of cleavage 4803 rfu/s/mg compared to 2314 and 2462), but this change was not replicated across the other two samples nor with trypsin-like or elastase-like proteases. A control sample was also run: one faecal sample was heated to 100°C for 5 min before protein was extracted and analysed following the same protocol as the other samples (Fig. S2). This sample showed no detectable protease activity across all three substrates. These results suggest that protease activity is stable in crude faecal samples for up to 72 hours at both room temperature and 4°C.

3.1.2. Protease activity is stable in whole faecal samples compared to extracted samples

To assess whether protease activity is better preserved when samples are stored as crude faecal samples compared to extracted protein, IBD faecal samples were processed as described in methods section 2.2 and the extracted protein in buffer solution stored at 4°C for 24 h along with the remaining unprocessed crude faecal sample. After 24 h, protein was extracted from the remaining crude faecal sample and protease activity of all samples assessed (using the three fluorescent substrates previously described). To determine whether storage in buffer solution would improve protease stability, protease activity levels were measured in samples stored as whole faecal samples compared to samples extracted in our optimised buffer conditions (Supplementary Results 1). However, these results instead showed increased activity levels for samples stored as crude faecal samples with a statistically significant difference between both groups when measuring chymotrypsin-like and trypsin-like activity ($p = 0.0312$ and $p = 0.0312$) and a numerical, but non-significant difference for elastase-like activity ($p = 0.313$; Fig. 3). Larger percentage decreases in activity seemed to occur in samples with higher overall protease activity levels although did occur at all levels.

3.2. Impact of sample processing on protease activity

3.2.1. Bead-beating has no significant effect on protease activity

To determine if increased bacterial and host cell lysis during protein extraction would significantly impact overall protease activity, we assessed the effect of adding a bead-beating step into the extraction protocol detailed in section 2.2. No significant difference was seen for any of the three substrates used between samples where bead-beating was added to the protein extraction process, compared to no bead-beating (Fig. 4), suggesting the predominant source of protease

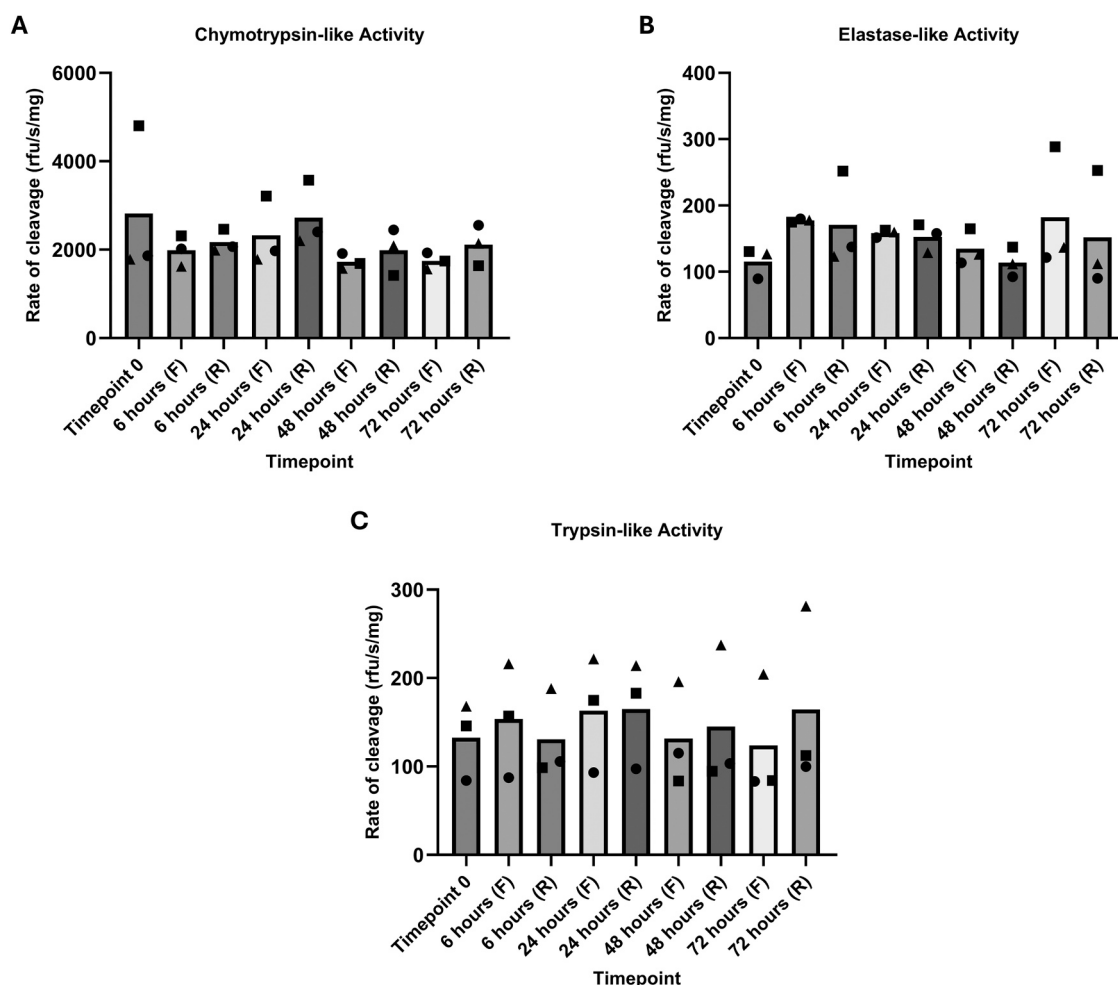


Fig. 2. The effect of differing storage conditions and duration prior to freezing at -80°C on protease activity. (F) = refrigerator storage at 4°C prior to freezing, (R) = storage at room temperature ($20\text{--}25^{\circ}\text{C}$). Individuals identifiable by different point shapes. A – chymotrypsin-like activity across varying storage conditions prior to freezing at -80°C ($p = 0.433$), B – elastase-like activity across varying storage conditions ($p = 0.372$), C- trypsin-like activity across varying storage conditions ($p = 0.480$).

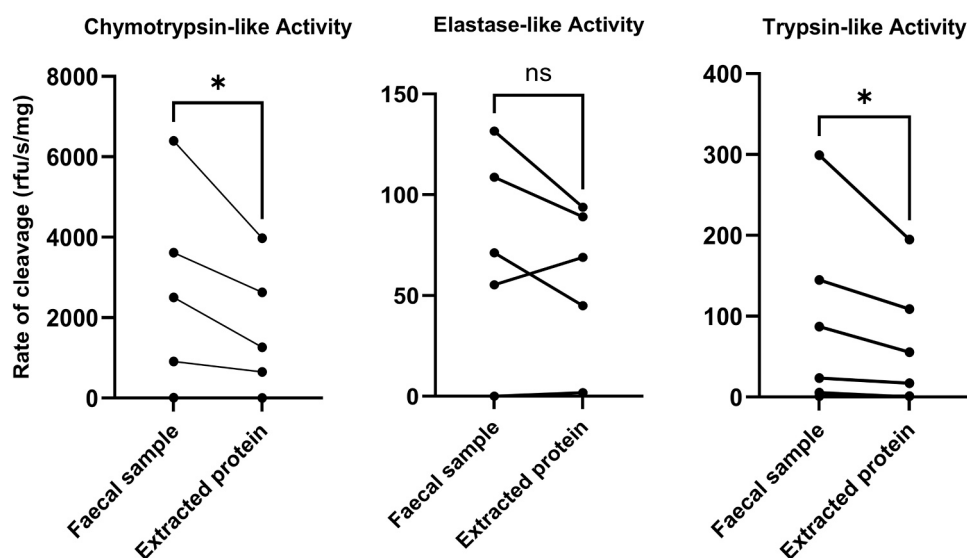


Fig. 3. Protease activity compared between samples stored for 24 hours at 4°C as whole faecal samples to extracted protein in buffer. Significant differences seen for chymotrypsin and trypsin-like activity ($p = 0.0312$ and $p = 0.0312$) and a numerical but non-significant difference for elastase-like activity ($p = 0.313$).

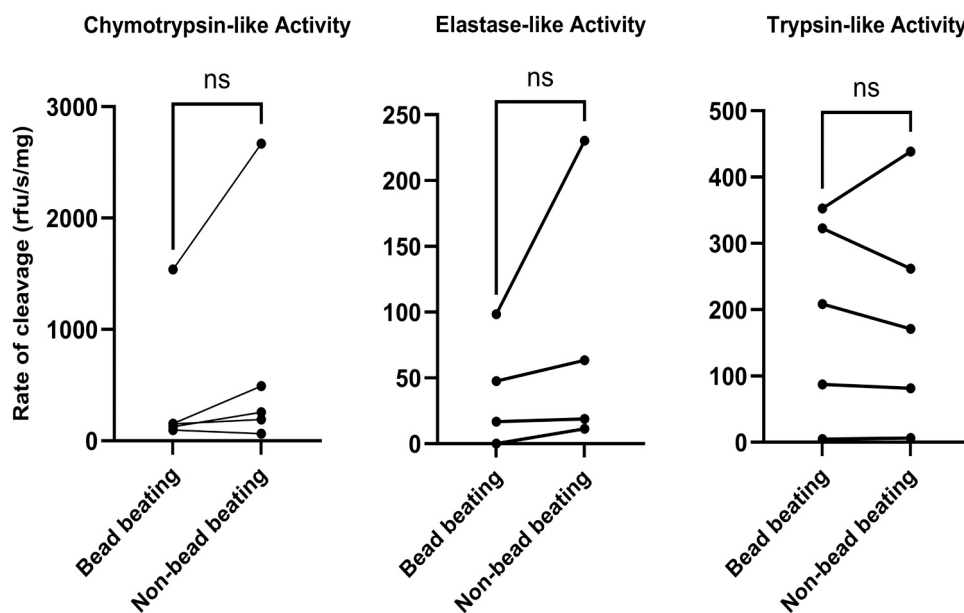


Fig. 4. The effects of bead-beating on protease activity compared across all three substrates. Chymotrypsin-like $p = 0.125$, Elastase-like $p = 0.125$, Trypsin-like $p = 0.812$.

activity is secreted luminal proteins rather than intracellular proteases released by cell lysis during beating. We therefore conclude that the addition of bead-beating does not significantly enhance serine-like protease activities.

3.2.2. Protease activity is more stable when stored on ice during sample processing

To investigate the effect of different handling temperatures during sample processing on protease activity; samples from active IBD patients were divided, after initial vortexing with extraction buffer and centrifugation, into two aliquots. One aliquot was stored on ice for 4 h and the other was left at room temperature for the same period. A four-hour period was used to approximate the time samples would typically spend out of a freezer for experiment preparation. Across all three substrate types there was a reduction in average protease activity when comparing samples kept on ice ($0-4^{\circ}\text{C}$) throughout processing compared to samples kept at room temperature ($20-25^{\circ}\text{C}$; Fig. 5). This difference was statistically significant in the case of chymotrypsin-like activity ($p = 0.0078$) but only numerically different for elastase and trypsin ($p = 0.203$, $p = 0.203$). This highlights the importance of

keeping samples cold whilst processing.

3.3. Summary of results

Chymotrypsin-like, trypsin-like and elastase-like activity were stable for up to 72 h when faecal samples were stored as crude samples at either room temperature or 4°C . However, protease activity from samples stored as extracted protein declined more quickly, consistent with previous studies [11]. Faecal samples are complex and consist of a wide variety of constituents with the ability to influence protease activity. Our results lead us to conclude that gut proteases are potentially less vulnerable to degradation whilst remaining in this environment suggesting some protective effect of remaining within a faecal matrix. We suggest that for future biomarker assays aimed at protease activity, samples should be collected and stored as whole faecal samples, before immediate processing prior to protease assay. Importantly, the stability of protease activity demonstrated up to 72 h is similar to that of faecal calprotectin, the most widely used current faecal biomarker in IBD [10].

The substrate-based techniques used in this study are broad measures of serine-like protease activity. It could be postulated that persistent

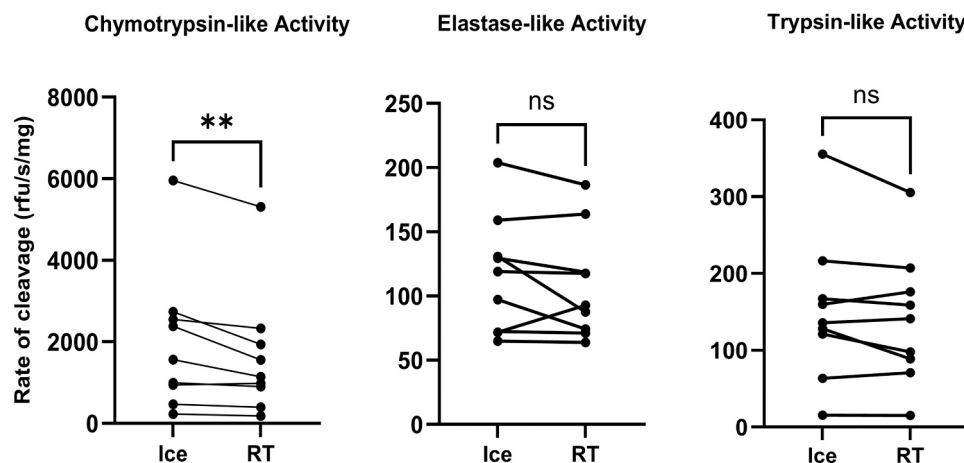


Fig. 5. The effect of handling extracted protein on ice compared to room temperature (RT) for 4 hours across three substrates. Chymotrypsin-like $p = 0.0078$, elastase-like $p = 0.203$, trypsin-like $p = 0.203$.

activity measured in samples stored for longer periods prior to freezing, may result from bacterial growth in the samples producing new associated protease activity, rather than the original source of activity present in the sample. More comprehensive profiling and identification of proteases would be needed to confirm if this is the case. However, given that in real world practice it is often not possible or practical for all faecal samples to be immediately frozen at -80°C , these results allow us to suggest that if faecal samples are stored as whole faecal samples for a few hours at room temperature or refrigerated, no significant reductions in overall protease activity are observed. Additionally, whilst the use of proteases as therapeutic targets in gastrointestinal disease has been considered previously, their use as biomarkers has not been widely evaluated [1]. Whilst further work on this area is needed, these results suggest the presence of several gut proteases that are stable in crude faecal samples for up to 72 h, potentially making them suitable for use as diagnostic biomarkers.

With regards to extraction methods, protease activity levels are higher when using buffers with $\text{pH} > 7$ and Tris based buffers (Supplementary Results 1). Given pH levels in the healthy colon tend to range between 6.1–7.5 we used a Tris based buffer at a pH of 7.5 to keep samples as close to biological pH as possible [18]. Furthermore, bead-beating did not significantly alter protease activity levels across all three enzyme substrates. These data suggest that protease activity seems to be predominantly driven by secreted proteins rather than from the intracellular proteins released following cell lysis induced by bead beating. The study from Morris and Marchesi showed an increase in protease activity from samples where bead-beating had been performed, which is in contrast to our results [11]. In their paper an azocasein substrate was used to assess activity which is a more general measure of protease activity than the substrates used in our experiment [19]. We observed a non-significant increase in trypsin for samples where bead-beating was performed and it is therefore possible that their observed increase with bead-beating was reflective of increased trypsin activity observed with bead-beating. Our data also shows the importance of processing samples on ice following protein extraction where possible due to the reduction in activity seen in samples left at room temperature for only four hours compared to those kept on ice.

3.4. Recommendations

These results therefore lead us to suggest the following:

- Store faeces as crude samples at -80°C and perform faecal protein extraction at the time of protease activity analysis
- For protein extraction use Tris-based buffers at higher pH (>7) (Fig. S1).
- The addition of bead-beating is not required to increase overall protease activity
- Keep samples cold during extraction and assay preparation.

4. Conclusion

Our study shows that overall FP activity is stable when samples are stored as whole faecal samples up to 72 h. Activity appears to decline more quickly when samples are stored as extracted protein. We suggest that the optimal approach is to store samples as crude faecal samples for as long as possible prior to analysis. Whilst it seems sensible to transfer faecal samples to deep freezing at -80°C or -20°C as soon as is practically possible, these data show a delay of up to 72 h does not significantly impact protease activity when compared to samples frozen within one hour. Additionally, these data show that when extracting proteins a Tris based buffer, at higher pHs, may be superior to PBS based buffers, that bead-beating of faecal samples does not significantly increase protease activity and that samples should always be kept on ice whilst handling.

A limitation of this study is the small sample size used in some

experiments. All samples were from patients with active IBD, and experiments assessed sample storage and processing using controlled conditions meaning that biological variability is smaller than if patients with more varied medical conditions were included or if experimental conditions were more heterogeneous. When analysing the effect of different storage conditions (Fig. 2), each patient sample was analysed at nine distinct time points, generating a total of 27 datapoints. The repeated measures increased power, therefore mitigating the limitations of the small sample size. Strong correlations between technical replicates were observed in all experiments demonstrating the reliability of the protease assays and the reproducibility of our findings. However, future studies with larger cohorts will be valuable for further validation and generalising these findings in broader patient cohorts.

Together our findings indicate the potential of protease activity as a clinically feasible biomarker assay for IBD. This study focused on broad-spectrum serine-like protease activity and our results will inform the development of standardised protocols for the collection and processing of faecal samples for accurate protease activity analysis. Future work should attempt to identify individual protease biomarker candidates using advanced analytical methods such as activity-based proteomics and assess their optimal stability and extraction methods.

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CRediT authorship contribution statement

Robert W. Perry: Writing – original draft, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Frederic Buemi:** Writing – review & editing, Supervision, Methodology, Investigation. **Xinyu Guo:** Investigation, Data curation. **Shiva T. Radhakrishnan:** Writing – review & editing, Resources, Investigation. **Sharmili Balarajah:** Writing – review & editing, Resources. **Lucy C. Hicks:** Writing – review & editing, Supervision. **Julian R. Marchesi:** Writing – review & editing, Supervision. **Horace R.T. Williams:** Writing – review & editing, Supervision, Funding acquisition. **Rachael Barry:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Shiva T Radhakrishnan reports a relationship with Alfasigma USA, Inc. that includes: speaking and lecture fees. Shiva T Radhakrishnan reports a relationship with Pfizer that includes: travel reimbursement. Shiva T Radhakrishnan reports a relationship with Ferring Pharmaceuticals Inc that includes: travel reimbursement. Robert W Perry reports a relationship with Ferring Pharmaceuticals Inc that includes: travel reimbursement. Sharmili Balarajah reports a relationship with Bowel Research UK that includes: funding grants. Sharmili Balarajah reports a relationship with Ferring Pharmaceuticals Inc that includes: travel reimbursement. Sharmili Balarajah reports a relationship with Dr. Falk Pharma AG that includes: travel reimbursement. Horace R T Williams reports a relationship with Pfizer that included: advisory. Horace R T Williams reports a relationship with Takada that include: travel reimbursement. Julian R Marchesi reports a relationship with EnterBiotix Ltd that includes: consulting or advisory. Julian R Marchesi reports a

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jpba.2025.116924](https://doi.org/10.1016/j.jpba.2025.116924).

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