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Sheep intelectin-2 co-purifies with the mucin Muc5ac from gastric mucus

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ABSTRACT

Secretion of gastric mucins plays an essential role in host protection, and modifications in mucus properties are characteristic of the protective immune responses to pathogens. This study describes the purification and characterisation of sheep gastric mucins, and identification of those proteins that co-purify with mucins, with the potential to modify mucus properties. Gastric mucus was collected and pooled from four abattoir sheep and separated by CsCl density gradient centrifugation. Proteomic analysis of the mucin-containing fraction indicated the presence of gastric mucin (Muc5ac) and several co-purifying proteins, including intelectin-2 (Itln2). Further experimentation indicated that a combination of denaturation and reduction was required to fully release Itln2 from gastric mucin. A putative correlation was found between mucin-bound intelectin concentration and rheological properties in further sheep gastric mucus samples. In conclusion, this study provides the first characterisation of sheep gastric mucins and their purification partners, revealing potentially important mucin-intelectin interactions.

1. Introduction

Most mammalian mucosal epithelia are covered in a protective mucus layer, which performs a number of essential roles, such as prevention from dehydration, lubrication and protection from pathogens. The predominant constituents of mucus are mucin glycoproteins, which are large, highly glycosylated, molecules of $\sim 2 \times 10^6$ Da which polymerise *via* disulphide bridges and are capable of forming a gel porous to small molecules (Ali and Pearson, 2007). The nature of the mucus layer and mucin gene expression is highly tissue specific, with for example, the Muc2 variant being predominant in the intestine, and the Muc5ac variant being the major form expressed in the upper airways and stomach (Audie et al., 1993).

It has long been known that gastrointestinal helminth infection induces major inflammatory changes at the epithelial layer, including significant changes in mucus secretion and properties (Miller, 1987). The nematode parasites *Teladorsagia circumcincta* and *Haemonchus contortus* both target the gastric (abomasal) niche in sheep, which respond with mucosal hypertrophy and an altered glycosylation profile (Hoang et al., 2010a,b). Furthermore, various effector proteins such as galectin-15 (also known as ovgal11) (Dunphy et al., 2000) and intelectin-2 (French et al., 2009) have been shown to be up-regulated in the sheep gastric epithelia in response to parasite infection, and are hypothesised to interact with gastric mucins. These putative interactions require to be investigated both *in vitro* and *in vivo*, which will necessitate purification of sheep gastric mucins.

It was our original intention in this study to purify sheep gastric mucins using standard protocols, so that specific mucin – effector protein interactions could then be studied *in vitro*. However, we discovered that mucin fractions were already associated with various putative effector proteins, and have documented this finding here, with particular focus on intelectin – mucin interactions.

2. Materials and methods

2.1. Sheep mucin preparation

In order to purify gastric mucins, the gastric mucosae (abomasa) of four sheep were first scraped with a microscope slide to remove mucus, and mucus scrapings were pooled. Mucin purification was performed as previously described (Jatkar et al., 2010). Briefly, the pooled scrapings were suspended in 10 vol of protease inhibitor buffer (Jatkar et al., 2010) and homogenised at full speed for 1 min. The homogenate was centrifuged at 13,000*g* for 1 h at 4 °C. The supernatant was adjusted to a density of 1.42 g/ml by the addition of solid CsCl. This was then centrifuged at 100,000*g* for 48 h at 4 °C to produce a density gradient. After centrifugation the gradient was fractionated into 8 equal fractions. After dialysis against distilled water, mucin and protein distribution within the gradient was determined by assaying for glycoprotein using the Periodic Acid Schiff's (PAS) assay (Mowry, 1963) and protein using

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the BioRad Protein Microassay (BioRad, Hemel Hempstead, UK). The fractions were then lyophilised and stored at −20 °C until required. Subsequently, 13 individual sheep abomasa were scraped and density gradients run and processed separately. None of the sheep used showed signs of any gross abomasal pathology or visible worms.

2.2. Anti-intelectin western blotting

Dialysed and lyophilised density gradient fractions were reconstituted with deionised water at 2.5 mg/ml by total weight. Ten microlitres of each fraction was separated by SDS–PAGE (12% T), then blotted and probed with a chicken anti-intelectin antibody as previously described (French et al., 2007).

2.3. Proteomic analysis of mucin fractions

Fifteen microlitres of mucin fraction F6 was separated by reducing SDS–PAGE (12% T) in a single lane and Coomassie blue stained (Imperial Protein Stain, Perbio Science UK, Cramlington, UK). A similar sample was run under non-reducing conditions. From the reduced sample, the resolving gel was divided into 22 equal slices and subjected to in-gel trypsin digestion, as previously described (Pemberton et al., 2010). From the non-reduced sample, the top 2 mm of the stacking gel was excised and subjected to in-gel digestion, as above. Tryptic digests were analysed by LC-MSMS, as described previously (Pemberton et al., 2010).

2.4. Re-purification of mucin-containing fractions

Mucin containing fraction F6 was subjected to repeat CsCl density gradient centrifugation, as described above (control gradient) and also in the presence of (a) 4 M guanidinium HCl (GuHCl) and (b) 0.2 M 2-mercaptoethanol. Mucin fractions were then subjected to anti-intelectin western blotting as described above.

2.5. Semi-quantitative Itln densitometry in density gradient fractions

Lyophilised density gradient fractions from 13 separate sheep were reconstituted in deionised water to 1 mg/ml final concentration. For each individual sheep, all 8 density gradient samples were run on the same reducing SDS–PAGE gel with the equivalent of 1 μg of sample per lane. A sample of sheep 1, fraction 1 was also run on all gels. The images for each western blot were loaded into ImageJ (http://rsbweb.nih.gov/ij/) for densitometry analysis. Each result was expressed relative to the sheep 1, fraction 1 sample run on that gel. Additionally, a sample of fraction 6 from all sheep was run on a single western blot and the Itln band intensity for each sample obtained by densitometry.

2.6. Rheological analyses of sheep mucus samples

Small deformation measurements were carried out using a Bohlin CV050 rheometer fitted with serrated parallel plates, 40 mm in diameter, set at a gap of 1 mm and operating in oscillatory mode. Mucus gels scraped from 13 sheep gastric mucosa were analysed. An amplitude sweep at 1 Hz was first carried out over the stress range 0.03–100 Pa, in order to determine the shear independent plateau of the elastic modulus G′ and the shear stress required for gel breakdown. Subsequent frequency sweeps were carried out at stress/strain values in the shear independent plateau over a frequency range 0.1–3 Hz. From these sweeps, values for the phase angle (δ), loss and storage moduli G″ and G′ could be determined. Viscosity measurements were made using a Contrives low shear viscometer at 20 °C with 5 mg/ml mucin samples in PBS.

2.7. Correlation of intelectin densitometry with rheological parameters

The rheological parameters G′ and breakdown stress were correlated with Itln densitometry results using non-parametric Spearman correlation statistics (Instat, Graph Pad Software). The intelectin results used in the analyses were (a) combined F5–F7 densitometry values (total mucin-associated Itln) and (b) individual F6 values. Correlations were considered significant where the associated p-value was less than 0.05. The analysis was run twice: firstly restricted to only those sheep which had detectable amounts of Itln in their mucin fractions, and secondly using results from all sheep.

3. Results

3.1. Mucin preparation by density gradient centrifugation

Based on the glycoprotein assay, 86% of the PAS positivity was present in fractions 4–7 in the density range 1.40–1.50 g/ml; a density range characteristic of other mammalian gastric mucins (Starkey et al., 1974). When the dialysed/lyophilised CsCl density gradient fractions were analysed by SDS–PAGE (Fig. 1A), it was clear that the majority of high MW mucin material was present in fractions 5 and 6. The mucin fractions also contained a significant number of other protein bands. Anti-intelectin western blotting (Fig. 1B) indicated the presence of immunopositive bands in most fractions with the strongest signal in fractions 5 and 6.

3.2. Identification of proteins co-purifying with sheep gastric mucins

Mucin fraction 6 was chosen for proteomic analysis, which indicated the presence of Muc5ac, intelectin-2 (Itln2), galectin-4, gastrokine (18 kDa antrum mucosa protein) and lysozyme 4a (Table 1). Muc5ac was detected in 12 out of 22 slices, suggesting the presence of mucin fragments of varying sizes. Furthermore, the non-reduced high MW band excised from the top of the stacking gel was found to contain Itln2, in addition to Muc5ac. This suggested that Itln2 is present in association with mucins in a manner that is not readily disrupted in the presence of SDS but absence of reducing agent.

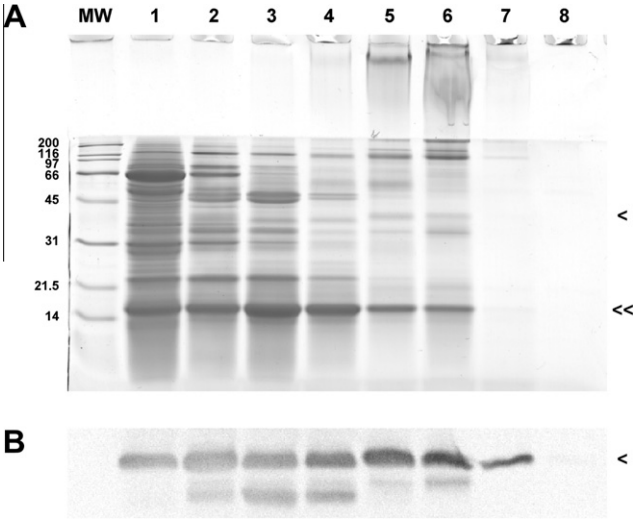

Fig. 1. Density gradient separation of sheep gastric mucus. Mucins separate in fractions 4–7. (A) SDS-gel (12% T) of CsCl density gradient fractions, prepared as described in Section 2. (B) Anti-intelectin western blot of CsCl density gradient fractions. Note presence of Itln positive bands in mucin fractions 5–7. The location of full-length Itln (<) and lysoyme (≪) are indicated.