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Partial purification and characterisation of sheep mucosal layer proteins

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ABSTRACT

The present study was undertaken to partially purify and characterise the sheep mucosal proteins. Cationic proteins were extracted from mucosal epithelium of sheep intestine, trachea and uterus. Proteins were partially purified by low pressure liquid chromatography (LPLC) and reverse phase high performance liquid chromatography (RP-HPLC). The extracts were subjected to 12.5% SDS-PAGE and 15% acid urea polyacrylamide gel electrophoresis for resolving into the constituent proteins. Different mucosal tissues yielded different concentrations of proteins, lowest from intestine and highest from uterus, which indicated presence of more cationic proteins in sheep uterus than that of sheep intestine. Protein extract of sheep intestine and sheep trachea was separated into 3 peaks each whereas sheep uterus into 4 peaks when subjected to low pressure liquid chromatography. In analytical RP-HPLC, sheep intestinal acid extracted proteins were separated into 6 peaks having elution time of 8.20, 8.41, 8.85, 9.14, 9.52 and 11, and 7 peaks were detected at 6.06, 8.11, 8.42, 8.85, 9.15, 9.47, and 11.30 min in acid extract of sheep tracheal mucosa. When mucosal proteins were subjected to SDS- PAGE 11 protein bands were detected (166.3 kDa to 7.9 kDa) in sheep intestine. 16 bands were detected (166.3 kDa to 9.3 kDa) in sheep uterus and 14 bands were detected (166.3 kDa to 9.3 kDa) in sheep trachea. 166.3 kDa, 117.2 kDa and 100.0 kDa proteins are found common in all the 3 tissues. Acid extract of sheep intestine, sheep uterus and sheep tracheal mucosal layers were separated into 11, 12 and 11 protein bands each respectively, when subjected to 15% acid urea PAGE.

Key words: Sheep, Mucosal proteins, LPLC, RP-HPLC, SDS-PAGE, AU-PAGE

Most of antigens that encounter immune system along life enter body through mucosal surfaces of respiratory, gastrointestinal and urogenital tract. These are the largest areas within body in-contact with external environment. Mucosal surfaces of respiratory, gastrointestinal and urogenital tract separate external environment from internal sterile environment and so represent a first line of defense system. This barrier faces environments rich in pathogens, which have developed effective mechanisms for colonisation of epithelial surfaces and invasion of mucosal tissues, but also harmless antigens such as food-air-borne antigens or commensal bacterial flora. The latter represent the vast majority of the encountered antigens and require an appropriate response characterized by either ignorance or active suppression. However, for the former, a robust immune response is needed. Under these influences, mucosae have developed a complex immune system, anatomically and functionally different from the systemic immune system, which is capable of mounting an immune response against pathogenic antigens while maintaining the required ignorance or active suppression against non-pathogenic antigens. Both animals and plants possess potent, broad-spectrum antimicrobial peptides, which they use to fend off a wide range of microbes, including bacteria, fungi, viruses and protozoa (Zasloff 2002). In small ruminants like sheep these mucosal layer proteins are still to be characterised, hence the present study was undertaken to characterise these mucosal proteins.

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MATERIALS AND METHODS

Tissues, viz. intestine, trachea and uterus, were collected from freshly slaughtered sheep in local slaughterhouse, early in the morning in cold chain, washed with ice cold PBS and were stored at –20°C overnight before processing at 4°C. Tissue were partially thawed and washed in 0.9% NaCl solution. The outer lining of each tissue (intestine, uterus and trachea) was stripped away from outer muscle wall by using glass slides, the linings were weighed, minced, and

homogenised well with 2 volumes of 10% acetic acid containing protease inhibitors (0.002% PMSF in 50% isopropanol, 1µl/ml and 10 mM EDTA, 1µl/ml) and were kept overnight at 4°C with continuous stirring for complete extraction of proteins. Tissue homogenates were subjected to ultrasonication, 4 cycles of 30 secs each at 80 amplitude. The extracts were centrifuged at 27000g for 15 min at 4°C and the supernatant were collected and stored frozen at –20°C in different aliquots till further use. The mucosal epithelial layers of intestine, trachea and uterus were prepared as per Pattanaik *et al*. (2011). Cationic proteins from the mucosal layer were extracted with 2 volumes of 10% acetic acid containing protease inhibitors (0.002% PMSF in 50% isopropanol, 1µl/ml and 10 mM EDTA, 1µl/ml) and were kept overnight at 4°C with continuous stirring for complete extraction of proteins. The tissue homogenates were subjected to ultrasonication, 4 cycles of 30 secs each at 80 amplitude. The extracts were then centrifuged at 27000g for 15 min at 4°C and the supernatant were collected and stored frozen at –20°C in aliquots till further use. Protein content of the acid extracts of the mucosal layers was estimated (Lowry *et al*. 1951). The lyophilised protein samples from different mucosal layers were reconstituted with 500 µl of phosphate buffer (pH 7.2). The reconstituted samples after passing through the membrane filter (0.45µm nylon acrodisc) were subjected for LPLC and RP-HPLC fractionation.

The filtered samples were applied to the sephadex G-25 column (30 cm × 1 cm) and was run using LPLC maintaining the flow rate of 1 ml/ min. The fractions were collected per minute using the fraction collector. Different peaks of proteins in the sample were detected at 280nm using UV detector. The fractions corresponding to the peaks were pooled together and were subjected to freeze drying and stored for further use. Before applying the sample, the column was properly washed with phosphate buffer (pH 7.2) for 45 min maintaining the flow rate of 1 ml /min. 20µl of the filtered samples (except sheep uterine acidic mucosal extract) were injected to the analytical column (C_{18} micro bondapack of size 7.8 × 300 with particle size 10 micron) and was run using HPLC system maintaining the flow rate of 1 ml/ min using the solvent system of 50% acetonitrile containing 0.1% TFA. The UV detector of the system was set at 254 nm to detect different peaks of proteins in the sample. The column was properly washed with solvent (50% acetonitrile containing 0.1% TFA) for 45 min maintaining the flow rate of 1 ml/min before injecting the sample.

SDS-PAGE in discontinuous buffer system was carried out to determine the electrophoretic pattern of proteins present in mucosal tissue extract (Laemmli 1970). The electrophoresis was run on separating gel of 12.5% acrylamide gel and staking gel of 4.5% acrylamide concentration using the mini gel electrophoresis apparatus. Proteins amounting to 40–80µg were loaded in each well. 10 µl of molecular weight marker ranging from 116 kDa to 14.4 kDa in molecular weight was also loaded for determination of molecular weight. Electrophoresis was conducted at room temperature at constant voltage of 120 volt till tracking dye reached the end of the gel. The gels were stained for 2 h with staining solution i.e. 0.125% coomassie brilliant blue R-250, then de-stained with several changes of de-staining solution i.e. 10% acetic acid and 40% methanol in de-ionised water. After thorough destaining the gels were stored in 7% acetic acid till analysis. The gels were placed in scanning chamber of densiometer. The molecular weight and RF values were determined taking protein marker as standard reference for molecular weight.

Acid urea PAGE of acid extracted mucosal proteins was performed as per Spiker (1980) using 15% gel. The freeze dried samples were dissolved directly in denaturing sample buffer and 20 µl of sample solution was carefully loaded into the well and electrophoresis was run at 100 volts till the tracking dye reaches the lower end of the gel. At the end of electrophoresis, the gel was immersed in staining solution for 4 h with gentle agitation and subsequently de-stained with several changes of de-staining solution i.e. 10% acetic acid and 40% isopropanol in de-ionised water. After thorough destaining the gels were stored in 7% acetic acid till analysis.

RESULTS AND DISCUSSION

The estimation of mucosal cationic proteins of sheep is presented in Table 1. Extractability of the proteins from different mucosal tissues was different, lowest being the sheep intestine (10.02±0.11) and highest in sheep uterus (15.42±0.16) which might be due to the presence of more acid soluble basic proteins in sheep uterus than that of sheep intestine. Diamond *et al*. (1991) observed that the extracts of bovine tracheal mucosa had in abundance, tracheal antimicrobial peptides (TAP) and later (1993) showed that the expression of TAP gene was localized only to the air way epithelial cells.

Protein extract of sheep intestine was separated into 3 peaks appeared at about 18 min, 28 min and 30 min of elution time after applied to the LPLC column at 10 min of washing

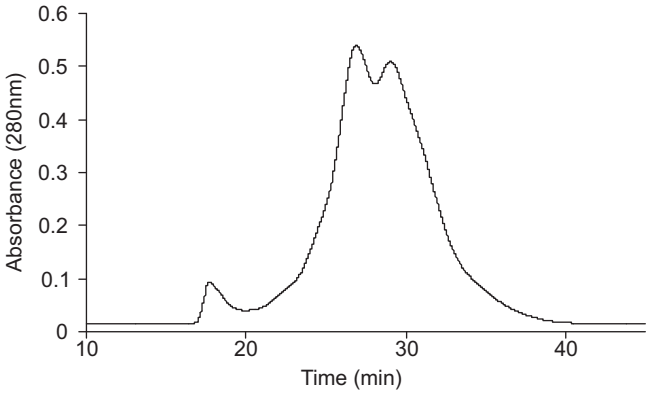


Fig. 1. Chromatographic pattern of Intestinal mucosal acid extract.

Table 1. Estimation of mucosal cationic protein (n=4)

Source	Amount of tissue collected from slaughter house (g)	Mucosal layer obtained after striping (g)	Amount of protein present in mg/ml of 10% acetic acid	Yield of cationic proteins (mg) per gram of mucosal epithelium
Sheep intestine	16.29±5.43	2.86±1.60	3.34±0.81	10.02±0.11[a]
Sheep trachea	13.92±2.38	0.49±0.11	3.89±0.73	11.67±0.26[a]
Sheep uterus	21.42±1.97	0.51±0.11	5.14±1.36	15.42±0.16[b]

Different superscripts in a column differed significantly (P<0.05).

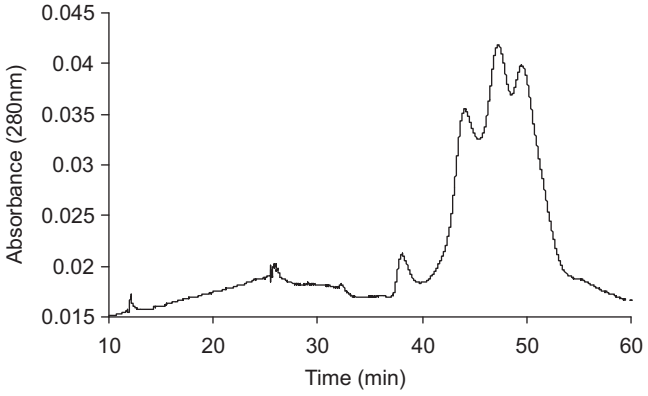


Fig. 2. Chromatographic pattern of uterine mucosal acid extract.

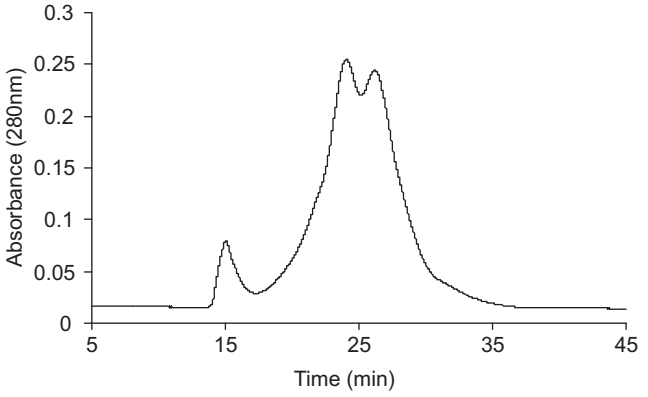


Fig. 3. Chromatographic pattern of tracheal acid extract.

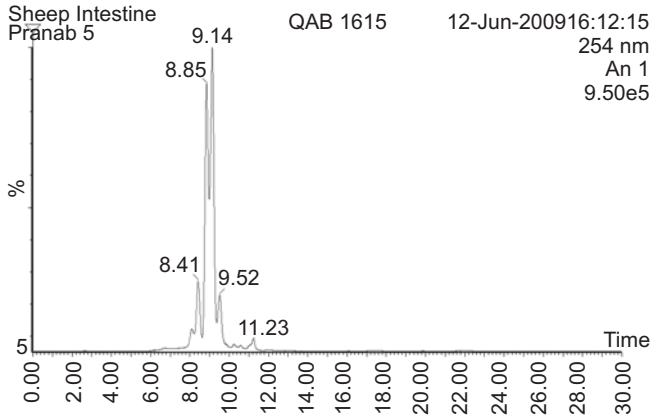


Fig. 4. Chromatographic pattern of intestinal mucosal acid extract.

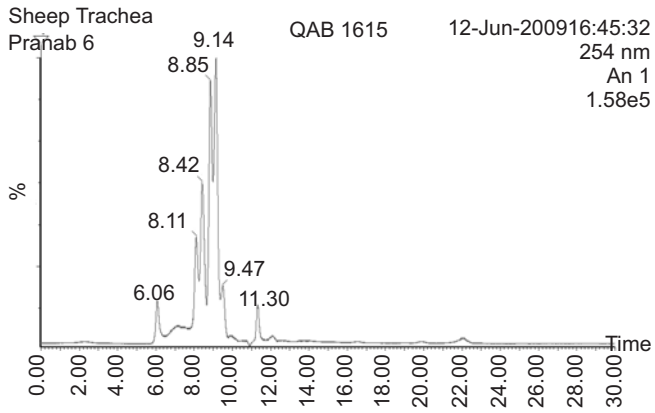


Fig. 5. Chromatographic pattern of tracheal mucosal acid extract.

(Fig. 1). Protein sample of sheep uterus was applied at 32 min of washing and separated into 4 different peaks. Peaks appeared at about 38, 44, 47 and 50 min of elution time (Fig. 2). Similarly the protein sample of sheep trachea was applied at 11 min and separated into 3 peaks appeared at about 16 min, 24 min and 27 min (Fig. 3).

Analytical RP-HPLC was performed for the mucosal acid extracted proteins to study the distribution of different types of proteins present in it. Sheep intestinal acid extracted proteins were separated into 6 peaks having elution time of 8.20, 8.41, 8.85, 9.14, 9.52 and 11 (Fig. 4). Seven peaks were detected at 6.06, 8.11, 8.42, 8.85, 9.15, 9.47, and 11.30 minutes in acid extract of sheep tracheal mucosa (Fig. 5). Sheep uterine mucosal extracts could not be subjected to RP-HPLC analysis. The RP-HPLC analysis of acid extract sheep mucosal layer is presented in Table 2. Different peaks in different mucosal extracts might be due to the presence of different type of proteins and their distribution in mucosal epithelial cells. The literature on chromatography of acid extracted mucosal proteins in sheep is scanty to discuss with but literatures available were on chromatography of acid extracted cationic proteins of PMN. Sharmah *et al*. (1993) dissolved the acid extracted cationic proteins of buffalo PMN cells into 3 peaks by using Sephadex G-50. Singh (1993)

Table 2. RP-HPLC analysis of acid extract sheep mucosal layer

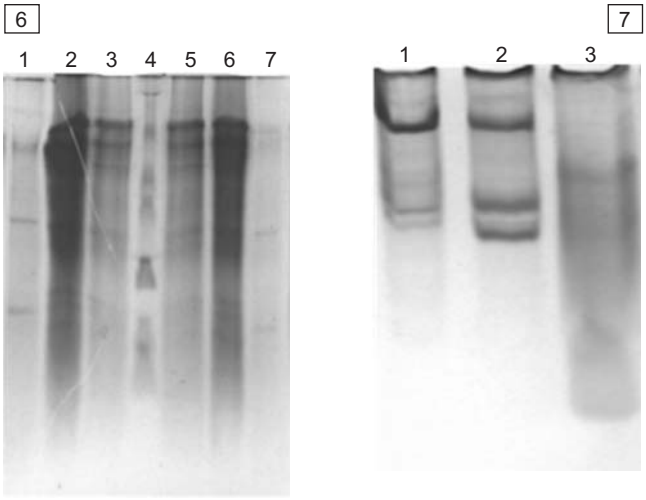
No. of peaks	Sheep intestine	Sheep trachea
1	8.2	6.06
2	8.41	8.11
3	8.85	8.42
4	9.14	8.85
5	9.52	9.15
6	11.23	9.47
7	-	11.30

Table 3. Comparative electrophoretic study of mucosal proteins in sheep

Band	Sheep intestine		Sheep uterus		Sheep trachea	
	Rm	Mol. Wt.	Rm	Mol. Wt.	Rm	Mol. Wt.
1	0.00	166.3	0.00	166.3	0.00	166.3
2	0.10	117.2	0.10	117.2	0.10	117.2
3	0.15	100.0	0.15	100.0	0.15	100.0
4	0.34	53.7	0.19	87.1	0.23	75.8
5	0.37	46.8	0.22	79.5	0.30	60.2
6	0.58	24.3	0.32	56.2	0.37	47.8
7	0.70	15.8	0.37	47.8	0.44	38.0
8	0.75	13.8	0.42	40.7	0.55	27.0
9	0.82	10.7	0.47	35.7	0.58	24.3
10	0.85	9.8	0.52	28.8	0.63	20.4
11	0.90	7.9	0.55	2 7.0	0.70	15.8
12	-	-	0.61	21.8	0.78	12.3
13	-	-	0.69	16.2	0.82	10.7
14	-	-	0.79	12.0	0.87	9.3
15	-	-	0.83	10.4	-	-
16	-	-	0.87	9.3	-	-

adopted GPC-HPLC based separation of cationic proteins of buffalo PMN granular acid extract and got 4 peaks. Similarly, Selsted *et al*. (1993) resolved the proteins of bovine PMN granules into 6 distinct peaks using Bio Gel P–60 columns and Evan *et al*. (1994) fractionated the avian heterophil granule extracts into 6 different peaks using Bio Gel P–10 column. In this study, Sephadex G-25 column was used for which less number of peaks were obtained i.e. 3, 4 and 3 peaks in sheep intestinal, uterine and tracheal mucosa respectively.

The SDS PAGE pattern of different mucosal proteins from intestine, uterus and trachea of sheep were compared. The comparative electrophoretic study of mucosal proteins in sheep is presented in Table 3. The protein marker was separated into 7 bands with relative mobility value ranging from 0.13 to 0.75 having molecular weight ranging 116 kDa to 14.4 kDa. In sheep intestine 11 bands were detected with molecular weight 166.3 kDa to 7.9 kDa, in uterus 16 bands with molecular weight 166.3 kDa to 9.3 kDa in, and in sheep trachea 14 bands were detected with molecular weight 166.3 kDa to 9.3 kDa (Table 3; Fig. 6). In all the three tissues, 166.3 kDa, 117.2 kDa and 100.0 kDa proteins are found



Figs–6–7. **6.** 12.5% SDS-PAGE pattern of sheep mucosal proteins (sheep intestine- lane 1 and 7, sheep uterus- lane 2 and 6, sheep trachea-lane 3 and 5. Marker-lane 4). **7.** Acid urea-PAGE pattern of mucosal proteins; lane 1-sheep trachea, lane 2-sheep uterus, lane 3-sheep intestine.

commonly. Although one-dimensional electrophoretic techniques revealed some of the major antimicrobial peptides within mucosal epithelial layers, the resolving power of these gels is not sufficient for the identification of other putative antimicrobial polypeptides. Tardy *et al*. (1995) through analysis of rat small intestinal mucosal lectin fraction revealed presence of 5 protein bands in the 14–20 kDa range with 2 prominent bands at 17 and 19 kDa by SDS-PAGE (named thereafter 17 and 19 kDa lectins). An iron-binding protein with an approximate molecular mass of 56 kDa was purified in human duodenal mucosa (Conrad *et al*. 1992).

The separation of mucosal proteins by 15% acid urea polyacrylamide gel electrophoresis is shown in Fig. 7. The comparative relative mobility of mucosal proteins in AU-PAGE is presented in Table 4. Acid extract of sheep intestine mucosal proteins were separated into 11 bands ranging in

Table 4. Comparative relative mobility of mucosal proteins in AU-PAGE

	Sheep intestine	Sheep uterus	Sheep trachea
Band	Rm	Rm	Rm
1	0.016	0.016	0.016
2	0.088	0.048	0.048
3	0.168	0.072	0.08
4	0.256	0.112	0.144
5	0.32	0.136	0.128
6	0.368	0.16	0.20
7	0.424	0.184	0.216
8	0.504	0.224	0.256
9	0.624	0.28	0.28
10	0.808	0.328	0.352
11	0.864	0.376	0.384
12	-	0.408	-

R_m of 0.016 to 0.864 (Table 4; Fig.7). Similarly, the mucosal proteins of sheep uterus were separated into 12 bands in R_m of 0.016 to 0.408. Sheep tracheal mucosal extract contained 11 protein bands ranging in R_m of 0.016 to 0.384. It is clear from the results that there were some proteins which are having similar mobility in all these tissues studied whereas, in some other proteins the relative mobility was different. In intestinal mucosa and uterine mucosa more cationic proteins were present than in tracheal mucosa.

From the above experiment, it may be concluded that in LPLC, the mucosal extract of sheep intestine, uterus and trachea were separated into 3, 4 and 3 peaks respectively. The mucosal extract of sheep intestine and trachea when subjected to RP-HPLC were separated into 6 and 7 peaks respectively. Sheep intestine mucosal extract was separated into 11 distinct protein bands having molecular weight ranging from 166.3 to 7.9 kDa, uterine mucosal extract into 16 protein bands ranging in molecular weight from 166.3 to 9.3 kDa and trachea was separated into 14 bands having molecular weight ranging from 166.3 to 9.3 kDa when subjected to 12.5% SDS-PAGE. The acid extracts of sheep mucosal membranes when subjected to 15% AU-PAGE, intestine mucosal proteins were separated into 11 bands, uterus into 12 bands and the tracheal mucosal extract into 11 protein bands. It was clear from the results that there were some proteins which are having similar mobility in all these tissues studied whereas, in some other proteins the relative mobility was different. In intestinal mucosa and uterine mucosa more cationic proteins were present than in tracheal mucosa.

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