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Proteomics and sequence analysis of a 30-kDa saliva protein of Indonesian local goat (*Capra hircus*) using MALDI-TOF/TOF-MS and related techniques

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Abstract: This study aimed to analyze and sequence a protein with molecular weight of approximately 30 kDa found in the saliva of the Indonesian local goat (*Capra hircus*) using matrix-assisted laser desorption/ionization-time of flight/time of flight-mass spectrometry (MALDI-TOF/TOF-MS) and related techniques. Saliva was collected from 50 healthy male and female local goats of 4–30 months old. The results showed that the average value of the total saliva protein was 669 ± 251.293 µg/mL, in the range of 350–1550 µg/mL. Mascot search and NCBI BLAST search revealed that the peptides were "PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like", with Mr = 26.656 kDa and pI = 4.57, which was 100% identical to *Ovis aries*. In addition to this, the peptide was also closely related to "TPA: common salivary protein BSP30 form d". Overall, the peptides belong to the BPI-protein superfamily, whose major roles are in innate immunity. Whether or not the identical state of the peptide in this study with those found in several other ruminants describes an evolutionary process that is common in ruminants still needs to be studied further.

Key words: Proteomics, Indonesian goat, *Capra hircus*, saliva, innate immunity

1. Introduction

Goats (*Capra hircus*) are one of the oldest small ruminants that humans domesticated about 10,000–10,500 years ago (1,2). They are known as the livestock of peasant farmers, since in general they are reared by farmers with very limited land ownership in rural areas, or in nonfarming communities where they are tethered and fed in backyards. According to FAO statistics, in 2013 there were over 975.8 million stocks of goats in the world (3). The population of goats in Indonesia was 18.5 million head in 2013 (4), spread out across the Indonesian archipelago. Types of goats in Indonesia are varied; some of them are noted as endangered species by the FAO, and those that are commonly found in the community are often referred to as local goats. In Indonesia, goats are also used as part of the government's efforts to increase farmers' income as well as to improve family nutrition.

Goats are widely spread even under poor natural conditions, thus indicating that the goat has great adaptability. Their adaptability has a particularly close relation to their dietary habits, which affect the durability of the goat to survive. Before entering the rumen, feed must first pass through the oral cavity. Saliva as a primary fluid in the oral cavity has a key role in the process of eating and digesting food, enabling goats to survive even in adverse conditions (5). Silanikove (5) reviewed that among ruminants in the desert, goats are the most efficient species in terms of grazing strategy. This is due to the morphologies of goats that have adapted in such a way; for example, desert goats have larger salivary glands (5). This illustrates that saliva plays a strategic role in feeding adaptation.

In the last few years, research on salivary proteomics in ruminants has increased, including that in goats (*Capra*) and sheep (*Ovis*) (6–9). Several proteins contained in ruminant saliva have been identified, although many still remain a mystery and are classified as 'predicted' proteins. These proteins seem to have much to do with the function of saliva as the first defense for the entrance of food into rumen. Lamy et al. (8) reported that at least 16 kinds of salivary proteins are found in the saliva of goats and sheep. Of the 16 proteins identified, there is a protein of 30 kDa that was found only in goat saliva. Proteins with the size of 30 kDa, according to the Saliva Proteome Project (http://fields.scripps.edu/public/project/saliva), are expressed as cathepsin H precursors. Cathepsin H is a member of the cathepsins, proteases ubiquitously expressed in animal tissues. Cathepsin H is known to be expressed in adult lungs and process pulmonary surfactant protein (10). However, it also plays a specific role in several types of

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tumors and cancers (11). The cathepsin H precursor found in the saliva of goats might be closely related to the cathepsin H expressed in the adult lungs and processing pulmonary surfactant protein. Therefore, this 30-kDa protein warrants more study.

Salivary proteins with a molecular weight of about 30 kDa in ruminants have attracted the attention of researchers in the past few decades. Rajan et al. (12) reported a salivary protein with a molecular weight of 30 kDa, which later became known as bovine salivary protein 30 (BSP30), is closely linked to the susceptibility of bloating in *Bos taurus* cattle (13,14).

This study aims to analyze and sequence a protein with the molecular weight of approximately 30 kDa found in the saliva of the Indonesian local goat using matrix-assisted laser desorption/ionization-time of flight/time of flight-mass spectrometry (MALDI-TOF/TOF-MS) and related techniques.

2. Materials and methods

2.1. Saliva collection

Whole saliva was collected from the oral cavity of 50 healthy male and female Indonesian local goats (age range: 4–30 months) using a plastic pipette, based on a previously published method with some modifications (15). All animals used in this study were kept under similar conditions. None of the female goats were pregnant or lactating. The goats were reared by peasant farmers collectively in the area near the Mataram University campus in Mataram, Lombok, Indonesia. All animals were fed native grass and leaves such as *Sesbania* spp. or jackfruit leaves, which were found around the pens. Sometimes they were also fed soybean dregs because of the tofu and tempeh household industries located in the area. The saliva samples were centrifuged at 3000 x *g* for 5 min at 4 °C to remove debris and remnants of feed from the oral cavity. The supernatant was collected and then sodium azide (NaN_3) as a preservative and phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich) were added to a final concentration of 0.02% (v/v) and 2 µmol/mL, respectively. The supernatant was then aliquoted at 500 µL each, frozen, and lyophilized. Following lyophilization, the saliva samples were resuspended in 100 µL of distilled water and kept at –20 °C until used for further analysis. Protein concentration was estimated using the Bradford reagent (Sigma-Aldrich) according to the manufacturer's procedure.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were subjected to separation in 12% SDS-PAGE using a vertical mini gel apparatus (Mini-V 8-10, GIBCO BRL), based on a previous method with several modifications (16). Individual samples were mixed with 5X concentrated SDS loading buffer (0.125 M Tris-HCl, pH 6.8; 2% SDS; 5% 2-mercaptoethanol; 20% glycerol with traces of bromophenol blue), with a ratio of loading buffer and protein solution of 5:1, and were boiled for 5 min and cooled. Equal amounts of proteins (10 µg in 10 µL) were loaded into the well of the 3% SDS-PAGE stacking gel. Simultaneously, a molecular mass protein marker (Broad-Way Dual Prestained Protein Marker, iNtRON Biotechnology) was also included in each gel for reference. Electrophoresis was carried out by using a running buffer [0.025 M Tris, 0.192 M glycine, 1% (w/v) SDS] at a pH of 8.3, with constant current of 80 V for 2 h.

Following electrophoresis, gels were fixed and stained for at least 2 h or overnight in a solution of 0.1% Coomassie brilliant blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid and destained with several changes of 10% (v/v) acetic acid.

2.3. Analysis of 30-kDa protein from SDS-PAGE gel using MALDI-TOF/TOF-MS

Identification of the 30-kDa protein was carried out according to a previous study (15). A stained band that corresponded to a 30-kDa relative mass was excised and sent to Proteomics International Pty. Ltd. (Nedlands, Australia) for MALDI-TOF/TOF-MS analysis. The protein samples were trypsin-digested and peptides were extracted according to standard techniques (17). Peptides were analyzed by MALDI-TOF/TOF-MS using a 5800 Proteomics Analyzer (AB Sciex). Spectra were analyzed to identify the protein of interest using Mascot sequence matching software (Matrix Science) with the Ludwig NR Database.

Search parameters used were MS/MS ion search, with carbamidomethyl as a fixed modification, oxidation as a variable modification, and monoisotopic with unrestricted protein mass as the mass value. Peptide and fragment mass tolerance were ±0.4 Da (mix missed cleavages = 1, number of queries = 40).

2.4. Statistical analysis

Quantitative data are expressed as mean ± standard deviation. Kruskal–Wallis nonparametric one-way ANOVA was carried out using PAST software (18) to analyze the appropriate data. Qualitative data are expressed visually and analyzed descriptively.

3. Results

3.1. Total protein in the saliva

The saliva was collected without any specific treatments from a total of 50 (38 male and 12 female) healthy local goats, with the age range of 4–30 months. The total protein of whole saliva of Indonesian local goats in this study was estimated (19) and the average value of the total protein was 669 ± 251.293 µg/mL, in the range of 350–1550 µg/

mL. For the average values of saliva total protein calculated based on age groups (months) of 4–12 (A), 12–24 (B), and >24 (C), results are given in Figure 1. No significant difference ($P > 0.05$) was observed among the three groups.

3.2. Salivary protein profile

Representation of the protein profile of Indonesian goat saliva is presented in Figure 2. It can be seen clearly that in the whole saliva of Indonesian local goats, a protein with a molecular mass around 30 kDa is predominant.

3.3. Identification of 30-kDa protein from SDS-PAGE gel

The predominant band with a molecular mass of 30 kDa was excised and analyzed by MALDI-TOF/TOF-MS, and from the Mascot search results there were two main proteins from the Ludwig NR Database that matched the excised 30-kDa band, i.e. the peptide with number W5P8S5 with Mr of 26.656 kDa and pI of 4.57, and W5P915 with Mr of 26.299 kDa and pI of 4.81. From the nominal mass (Mr) of both peptides, it can be seen clearly that the results were different from the predicted 30-kDa molecular weight of the excised band in this study.

Sequence similarity of W5P8S5 in an NCBI BLAST search of W5P8S5 against NR and W5P915 in an NCBI BLAST search of W5P915 against NR gave results that the protein was predominantly "PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like". This protein belongs to the bactericidal permeability-increasing protein (BPI) superfamily (20).

From the two proteins, W5P8S5 gave the highest score identity (100%) with the excised band in this study, though it was not from *Capra hircus* but instead *Ovis aries* (Accession No. gi|426241975|XP_004014855.1). Therefore, the Mascot hit was focused on W5P8S5 and the results are presented in the Table. In addition to "PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like", the excised 30-kDa band in this study showed identity with "short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like" of *Bos taurus* with 81% identity. With "TPA: common salivary protein BSP30 form d [*Bos taurus*]" there was 60% identity (Table).

4. Discussion

Goat is one of Indonesia's germplasms that have high economic value, particularly in rural areas. There are two main goat types in Indonesia, namely goats for meat and goats for milk production, the goats for meat being

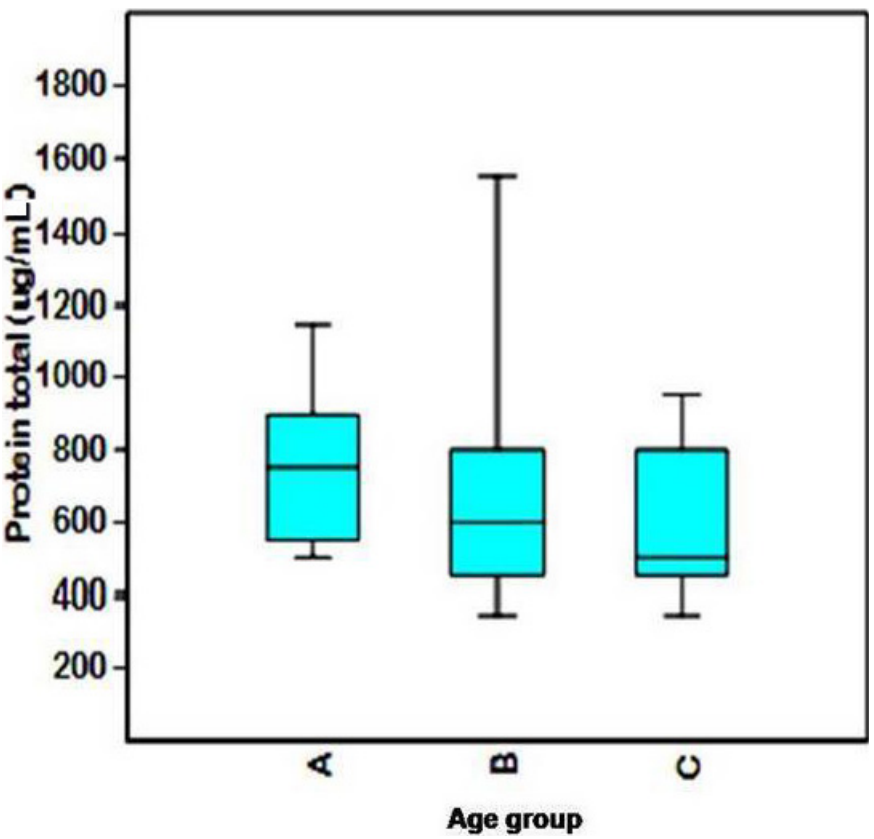

Figure 1. Values of total salivary protein concentration in three age groups of Indonesian local goats: 4–12 months (A), 12–24 months (B), and >24 months (C).

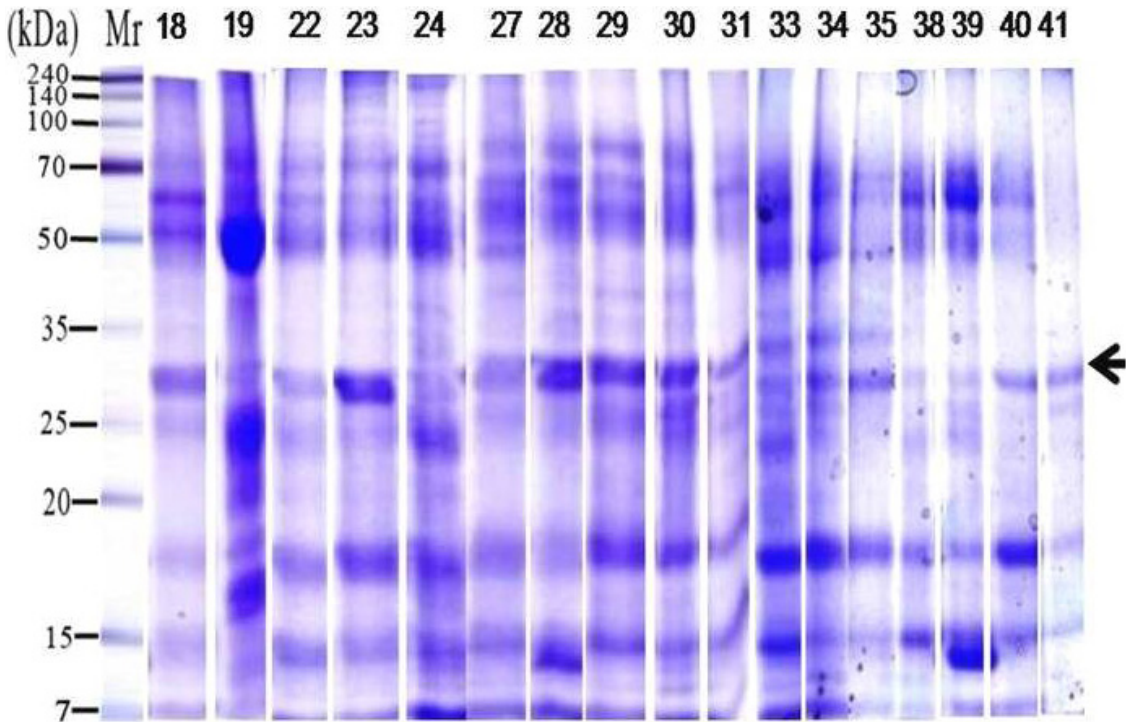

Figure 2. Representation of one-dimensional SDS-PAGE of 12% gel patterns of local goat saliva. Mr, Molecular weight standards, Broad-Way Dual Prestained Protein Marker (iNtRON Biotechnology, #24084). The 30-kDa band (arrow) was the proteomic target in this study. The bands were cut and used for MALDI-TOF/TOF-MS analysis. The numbers above the lanes represent the codes of goats used in this study.

more dominant due to geographical conditions and the habit of farmers liking this type of goat more. Studies on goats are heavily focused on the aspects of management and socioeconomic details as well as on feeding trials. Conversely, studies related to salivary proteomics are still very limited. This is understandable because the results obtained are still not able to be felt directly by farmers. However, such study is necessary to lay down a solid foundation for the future of the basics related to evolution, physiology, and immunology of ruminants, including goats.

Several studies over the last decade mainly focusing on saliva were especially seeking treatment methods for the noninvasive examination of animals, in order to reduce the suffering of the animals. In this study salivary proteomics of local goats have also been examined. The data obtained showed that the average of the total salivary proteins was 669 ± 251.293 µg/mL, in the range of 350–1550 µg/mL. These results are still within the range of the values of total salivary proteins in goats reported by Lamy et al. (7), i.e. 30–2000 µg/mL. We also analyzed the average value of total salivary proteins based on three age groups, A (4–12 months), B (12–24 months), and C (>24 months). Although there were indications of a decrease in the average of total salivary protein with the increase of age (Figure 1), the trend was not statistically significant ($P > 0.05$). Data on the relationship of age and salivary protein concentration in goats are still limited. Compared to data from humans, showing a positive correlation between age and protein salivary concentration (21,22), the tendency obtained in this study was opposite to that in humans. It is difficult to explain our data; many things still need to be researched with a better experimental design.

The results of one-dimensional SDS-PAGE analysis in this study (Figure 2) indicate that there are several bands of protein, as reported by Lamy et al. (7). The patterns of protein bands showed by these saliva samples remained relatively constant between individual goats. Interestingly, the band with a molecular weight of about 30 kDa existed in all age ranges of local goats used in this study. A similar band according to Lamy et al. (7) was only found in goat saliva and was not found in the saliva of sheep (*Ovis aries*). This suggests that salivary proteins with a molecular weight of 30 kDa have an important role in local goat, at least in this study. Salivary proteins with a molecular weight of 30 kDa in goats, according to the Proteome Project (http://fields.scripps.edu/public/project/saliva), are expressed as cathepsin H precursors. Cathepsin H is a member of the cathepsins, the proteases ubiquitously expressed in animal tissues. Cathepsin H is known to be expressed in

Table. Mascot search results of protein spot (30 kDa, from local goat saliva) identification by MALDI-TOF/TOF-MS.

No.	Description	Max. score	Query cover (%)	Identity (%)	Accession
1	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like [*Ovis aries*]	437	100%	100%	gi|426241975|XP_004014855.1
2	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like [*Capra hircus*]	387	94%	94%	gi|548491154|XP_005688521.1
3	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like [*Pantholops hodgsonii*]	388	100%	89%	gi|556723126|XP_005957388.1
4	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2B-like [*Pantholops hodgsonii*]	370	100%	87%	gi|556723124|XP_005957387.1
5	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2B-like [*Capra hircus*]	360	95%	87%	gi|548491803|XP_005688714.1
6	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like [*Bubalus bubalis*]	355	95%	87%	gi|594043603|XP_006046316.1
7	Short palate, lung, and nasal epithelium carcinoma-associated protein 2A precursor [*Bos taurus*]	353	95%	86%	gi|108796053|NP_777228.2
8	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2B-like [*Pantholops hodgsonii*]	355	100%	85%	gi|556769020|XP_005979720.1
9	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2B-like [*Ovis aries*]	352	94%	88%	gi|426241319|XP_004014539.1
10	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A isoform X1 [*Bos taurus*]	352	95%	85%	gi|528973356|XP_005214563.1
11	PREDICTED: LOW QUALITY PROTEIN: short palate, lung, and nasal epithelium carcinoma-associated protein 2B-like [*Bison bison bison*]	343	95%	80%	gi|742182792|XP_010853307.1
12	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2B-like isoform X1 [*Bubalus bubalis*]	342	95%	81%	gi|594105753|XP_006075819.1
13	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2B-like [*Bos mutus*]	340	95%	81%	gi|555969911|XP_005896979.1
14	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2B isoform X1 [*Bos taurus*]	338	95%	81%	gi|741947744|XP_010809671.1
15	Short palate, lung, and nasal epithelium carcinoma-associated protein 2B precursor [*Bos taurus*]	337	95%	81%	gi|27807385|NP_777227.1
16	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2B-like [*Ovis aries*]	263	73%	88%	gi|426258551|XP_004022873.1
17	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2B-like [*Ovis aries*]	246	100%	61%	gi|426241969|XP_004014852.1
18	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like [*Pantholops hodgsonii*]	244	100%	60%	gi|556718250|XP_005955023.1

Table. (Continued).

19	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2B-like [*Capra hircus*]	225	93%	60%	gi|548491116|XP_005688513.1
20	PREDICTED: LOW QUALITY PROTEIN: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like [*Bos taurus*]	219	93%	59%	gi|741888920|XP_010822589.1
21	TPA: common salivary protein BSP30 form d [*Bos taurus*]	208	84%	60%	gi|296481302|DAA23417.1
22	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2B-like [*Ovis aries*]	211	95%	50%	gi|426241971|XP_004014853.1
23	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like [*Bison bison bison*]	201	95%	55%	gi|742182787|XP_010853306.1
24	PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like [*Pantholops hodgsonii*]	194	95%	50%	gi|556718252|XP_005955024.1

adult lungs and process pulmonary surfactant protein (10). Since the protein in this study was from saliva, which was also closely related to the respiratory system, it may be close to the cathepsin H precursor. Differing from this, Rajan et al. (12) and Wheeler et al. (13,14) found a 30-kDa protein in *Bos taurus* saliva named BSP30, which was closely linked to the incidence of bloat.

Because the 30-kDa protein appears to have an important role in ruminants, we performed further analysis of the one-dimensional SDS-PAGE gel band associated with the protein of 30 kDa. The MALDI-TOF/TOF-MS analysis results showed that the gel pieces with a size of 30 kDa had an apparent mass of 26.66 kDa. This apparent mass is located in a position between 25 and 30 kDa, which was previously classified as the apolipoprotein A-I precursor (23,24). In line with this, Lamy et al. (7) also referred to the compounds for goat saliva. Interestingly, the results obtained from Mascot hits in this study showed that the majority of these proteins of 26.66 kDa are "PREDICTED: short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like" (Table), which is included in the BPI protein superfamily (25–27). In other words, the proteins or peptides in this study are distinct from apolipoprotein A-I. This shows how complex the saliva is. Furthermore, it must be noted from the results of this study that the proteins obtained from local goat saliva are 100% identical to those of *Ovis aries*, while its similarity against goat (*Capra hircus*) is only 94%. Although still "PREDICTED", the short palate, lung, and nasal epithelium carcinoma-associated protein 2A-like in this study is abbreviated as PLANEPC-AP 2A-like.

When examined more deeply, from the local goat salivary protein bands, there were at least two proteins that were not assigned as "PREDICTED", but rather directly named as "short palate, lung, and nasal epithelium carcinoma-associated protein precursor 2B" and "TPA: common salivary protein BSP30 form d", although the percentage identity was not 100% but rather 81% and 60%, respectively. It is confirmed that the local goat salivary protein that existed in the SDS-PAGE band with 30 kDa is from the BPI superfamily, which functions in innate immunity. Another interesting point is that this protein also appears to have similarities with BSP30d. As shown by Wheeler et al. (26), BSP30 comprises four forms, namely BSP30a, b, c, and d. BSP30a is a common form as found in whole *Bos taurus* saliva, while the forms of b, c, and d are scattered and vary among cattle. From the results of analysis of MALDI-TOF/TOF-MS on local goat saliva in this study, it is also interesting to study the exact nature of BSP30 in Indonesian local goats.

Acknowledgments

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