

Original Article

Complement C3 and anti-testosterone antibody in the 150 kDa Bali cattle (*Bos sondaicus/javanicus*) subordinate follicular fluid peptide observed using MALDI-TOF/TOF-MS

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ABSTRACT

Objective: This study aimed to elucidate the proteome profile of the 150 kDa protein isolated from the subordinate follicle of Bali cattle (*Bos sondaicus/javanicus*). Some researchers have revealed several factors in the follicular subordinate with a 150 kDa protein substance, which play important roles in the bovine ovulation.

Materials and methods: In the present study, subordinate follicles (~ 5 mm in diameter) were collected from 10 female Bali cattle from slaughterhouses in Taliwang, Sumbawa of West Nusa Tenggara Province, Indonesia. The follicular liquid was pooled; fractionated using SDS-PAGE 10%, the 150 kDa band was sliced and then analyzed using MALDI-TOF/TOF-MS.

Results: Mascot search results significantly revealed the presence of four species of proteins/peptides. Of the four peptides, two were predominant i.e. complement C3 and anti-testosterone antibody, which both were 100% identical to complement C3 and anti-testosterone antibody of *Bos Taurus* cattle.

Conclusion: Complement C3 and anti-testosterone antibody are present in the follicle fluid of *Bos sondaicus/javanicus* cows. These findings are novel in Bali cattle follicles.

KEYWORDS

Bali cattle; C3 complement; Proteomics; Subordinate follicle

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INTRODUCTION

The follicular fluid (FF) is produced during folliculogenesis, accumulates in the atrium of the ovarian follicle. It contains a variety of proteins or peptides that provide the micro environment for follicle development and oocyte maturation (Twigt et al., 2012; Zamah et al., 2015). It has been reported elsewhere, oocyte maturation is related with the presence of gonadotropins and steroids in the FF (Mason et al., 1994; Fortune et al., 2001; Revelli et al., 2009; Fahiminiya et al., 2010). In addition, abundance of factors related to oocyte maturation and folliculogenesis have also been explored in the FF such as inhibin A and B (Ocal et al., 2004), anti-Müllerian hormone (Mashiach et al., 2010), lactoferrin (Yanaihara et al. 2007), hyaluronan (Saito et al., 2000), leptin (De Placido et al., 2006), insulin-like growth factor-II (IGF-II) (Wang et al., 2006), and interleukin 8 (Malizia et al., 2010). Furthermore, Fortune et al. (2001) reviewed in cattle the presence of some growth factors and their receptors such as insulin-like growth factors (IGF), IGF binding protein-4 (IGFBP-4) play crucial role in differentiation or selection of dominant or subordinate follicles. Fahiminiya et al. (2010) successfully identified 18 proteins in the crude mare FF, and at least 113 in the enriched FF of mare follicular fluid during late follicle development. More recently, Fu et al. (2016) have successfully identified at least eleven differentially expressed proteins from small (<4 mm) and large (>8 mm) buffalo follicular fluid that are involved in serine protease inhibition, complement cascade system and oxidation protection system. From the findings, it was revealed that some proteins or peptides are of common proteins or factors while several are of species-specific.

Cattle have been known as important agricultural species to supply meat and milk. Study of the growth and development of cow follicular from various bovine breeds, including Bali cattle (*Bos sondaicus/javanicus*), therefore is challenged. Bali cattle are Indonesian native cattle, which has been developed from domesticated banteng, *Bos javanicus*, *Bos banteng* (Nijman et al., 2003; Mohamad et al., 2009). Bali cattle are also considered to have moderate to high (0.21–0.41) reproductive heritability, which means that Bali cattle have the potential to be genetically improved through selection programs, with good reproductive management practices (Gunawan et al., 2011).

Proteomic study of Bali cattle follicular fluid (FF) is still limited. In fact, understanding proteomic of FF in indigenous animals such as Bali cattle is important

because it involves the microenvironment for oocyte and granulosa cells development, which will then determine the development of the next generation. Furthermore, proteomics analysis of FF can provide helpful information for biomarkers discovery and treatment development (Veenstra et al., 2005).

Subordinate follicle is part of a cohort growing follicle 5-7 days prior to ovulation (Evans and Martin, 2000; Kanitz, 2003), and proteins with molecular weight of 150 kDa such as IGFBPs have been reported to implicate in a cohort growing follicle (Rivera and Fortune, 2003). Proteomic information regarding these is limited for Bali cattle. We therefore initially focus this study to elucidate the proteome profile of the 150 kDa protein isolated from the subordinate follicle of Bali cattle (*Bos sondaicus/javanicus*) by means of MALDI-TOF/TOF-MS.

MATERIALS AND METHODS

Follicular Fluid Collection and Preparation

Bali cattle follicular fluid (BcFF) was collected from 10 healthy Bali cattle cows ovaries based on corpus luteum (CL) size at a slaughter house in Town of Taliwang, Sumbawa Regency, West Nusa Tenggara Province, Indonesia. Follicles were dissected, measured and categorized as subordinate with the size of about 5 mm. Follicular fluid was aspirated and centrifuged at 3000xg for 10 min to remove cells and debris. The supernatants were pooled and total protein concentration was determined using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA) at a wavelength of 280 nm, and samples were kept at -70°C until processed.

SDS-PAGE and MALDI-TOF/TOF-MS analysis

All BcFF samples were thawed and when needed, they were diluted with phosphate buffered saline at pH 7.4 and brought to a 2 mg/mL concentration. SDS-PAGE was conducted under denaturing conditions in a discontinuous 1-D SDS-PAGE system using 10 % acrylamide according to Laemmli (1970). Each sample was mixed with loading buffer and loaded at 20 µg per well after boiling for 4 min at 100°C. As a molecular weight standard, a protein molecular marker also ran along with the BcFF samples according to the manufacture's instruction (Intron, Biotechnology). Electrophoresis was performed at a constant voltage of 100V until bromophenol blue front reached the bottom of the gel. The proteins were stained using Coomassie brilliant blue dye R-250 (Sigma, Aldrich), and then de-

stained to get optimum band image. Following de-staining process, the band corresponding to the molecular weight of around 150 kDa was sliced and put in a microtube containing 50 μ L deionized water before MALDI-TOF/TOF-MS was performed.

The sliced gels were treated according to [Bringans et al. \(2008\)](#). Briefly the samples were reduced, alkylated and trypsin digested, extracted and then analyzed by MALDI-TOF/TOF-MS using a 5800 Proteomics Analyzer (AB Sciex, Framingham, MA, USA). In order to identify the protein of interest, spectra obtained were analyzed using Mascot sequence matching software (Matrix Science, Boston, MA, USA) with Ludwig NR Database, as performed by [Depamede \(2013\)](#).

For the search and analysis process, MS/MS ion search was set up as suggested by the manufacture. Carbamidomethyl as fixed modification, oxidation as variable modification, and monoisotopic with unrestricted protein mass as mass value, were applied as search parameters. Furthermore peptide and fragment mass tolerance were ± 0.4 Da (mix missed cleavages=1, and number of quires=40) were set up throughout the searching and analysis process.

RESULTS AND DISCUSSION

This study aimed to identify proteins or peptides contained in the 150 kDa correspondence band of single dimension 10% SDS-PAGE of Bali cattle follicular fluid, BcFF using MALDI-TOF/TOF mass spectrometer. The 150 kDa band was sliced and analyzed by MALDI-TOF/TOF mass spectrometer combined with Mascot search program. The results of Mascot score histogram (**Figure 1**), show four main proteins observed, and the information of the proteins in more detail is presented in **Table 1**.

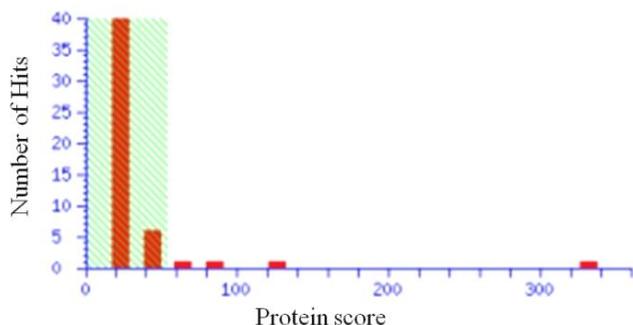


Figure 1. Representation of Mascot search results on BcFF150 in pooled Bali cattle follicular fluid. Mascot search program was set up according to manufacture default.

Based on the Mascot program, an individual ions scores > 53 indicate identity or extensive homology ($P < 0.05$). Of the four main proteins observed the highest protein score (332) was for Bovin Complement C3 of *Bos Taurus* origin. It means that most likely the BcFF-150 contained proteins that are homolog to the complement C3 of *Bos Taurus*. In addition to this there are two peptides that were named as Predicted Complement C3 of different animal species i.e. *Erinaceuseuropaeus* (western European hedgehog), and *Octodon degus*. These two species are of far distance species from Bali cattle, additionally the protein of *Erinaceuseuropaeus* origin has also been removed from NCBI Reference Sequence as a result of standard genome annotation processing. However, data on the protein score of the complement C3 or predicted C3 might possibly be related to the evolution nature of the organisms. We therefore, focused on two proteins (Complement C3 and anti-testosterone antibody) derived from *Bos Taurus*, which is part of the large ruminants group, but of a different breed from Bali cattle (*Bos sondaicus/javanicus*).

Complement C3 has a crucial role for the activation of the complement system particularly both classical and alternative complement pathways in the body ([Noris and Remuzzi, 2013](#)). The presence of Complement C3 has been reported in the human follicular fluid, and it was abundant in younger group as compared to older group ([Hashemitabar et al., 2014](#)). Complement C3 has also identified in the mare follicular fluid ([Fahiminiya et al., 2010](#)), in the buffalo ([Fu et al., 2016](#)); but is limited in the cows. Identification of Complement C3 in Bali cattle follicular fluid is therefore categorized as novel.

Complement C3 is a plasma protein that is abundantly synthesized by hepatocytes ([Zhou et al., 2016](#)), the presence of this protein in the follicular fluid mostly through the diffusion of the protein over the blood-follicle barrier, which its permeability is raised during follicle maturation ([Hanrieder et al., 2008](#)). C3 complement has also been reported to be synthesized and secreted by malignant ovarian epithelial cells ([Cho et al., 2014](#)), resulting in the activation of complement cascade in the tumor microenvironment and increases cancer cell proliferation, and metastasis. The regulation of C3 expression in malignant cells is not well understood, similarly, the involvements of the cascade in the innate immunity of follicular fluids still need to be explored. [Jarkovska et al. \(2011\)](#) have shown the role of complement in oocyte maturation. They demonstrated that activation of complement resulted in the deficiency of vascular endothelial growth factor (VEGF), in fact

Table 1. Identified of four major proteins contained in a 150 kDa sliced band of single SDS-PAGE of crude Bali cattle follicular fluid based on Mascot search.

No.	Accession No. ¹⁾	Protein name	Protein Score ²⁾	Nominal Mr (kDa)	Organism
1.	Q2UVX4	Bovin Complement C3	332	187.135	<i>Bos taurus</i>
2.	XP_007525030.1 ³⁾	PREDICTED: complement C3-like	128		<i>Erinaceus europaeus</i> (western European hedgehog)
3.	XP_004632856.1	PREDICTED: complement C3	75	186.571	<i>Octodon degus</i>
4.	440 or CAA44699	Anti-testosterone antibody	54	50.593	<i>Bos taurus</i>

¹⁾ Accession number is an accession number from the NCBI databases

²⁾ Protein score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event, it is based on NCBI database using the Mascot searching program.

³⁾ The record of this accession number has been removed from NCBI Reference Sequence as a result of standard genome annotation processing.

VEGF is needed for oocyte maturation. Their findings were in line with [Hashemitabar et al. \(2014\)](#) that the C3 complement concentration was found considerably higher in follicular fluid of fertilized oocyte compared to those of non-fertilized. Furthermore, [Jarkovska et al. \(2011\)](#) reported that in women with severe ovarian hyper stimulation syndrome, complement C3 was expressed differentially. In our study, the detection of complement C3 in BcFF whether related to the reproductive nature of Bali cattle ([Gunawan et al., 2011](#)), challenge the researchers to elucidate what the role of BcFF complement C3 is on the Bali cattle reproductive system.

In addition to complement C3, in this study, anti-testosterone antibody was also identified in the BcFF. The presence of immunoglobulins in the follicular fluid essentially has also been reported elsewhere in the follicular fluid of canine, human, porcine, and mare ([Fahiminiya et al., 2010](#)). Interestingly, information regarding the existence of immunoglobulin, particularly anti-testosterone antibody in cow follicular fluid, is rarely reported; but was identified in Bali cattle follicular fluid in this study. Testosterone, but not anti-testosterone antibody, has been reported since more than a decade to be able to stimulate transition of bovine follicle from primary to secondary follicle ([Yang and Fortune, 2006](#)). Recently, androgens (including testosterone), although is still in debatable, have been used as adjuvant for follicle maturation and ovulation induction ([Gleicher et al., 2011](#); [Garcia-Velasco, 2014](#)). It was also stated elsewhere that androgen excess can be disadvantageous to women's health and fertility ([Rosenfield and Ehrmann, 2016](#)).

We may therefore speculate that the anti-testosterone in the Bali cattle follicular fluid in a certain condition act as antagonist to down regulate testosterone in the follicular fluid for a homeostatic purposes. In this study, although the protein score of anti-testosterone antibody is quite low (*i.e.*, 54) which is close to the minimum significant score value of >53 , its presence in the Bali cattle follicular fluid is also interesting to be investigated further.

CONCLUSION

Proteomics analysis in this study revealed that the 150 kDa proteins isolated from the subordinate follicle of Bali cattle (*Bos sondaicus/javanicus*) consisted of four peptides, two of them were predominant *i.e.* complement C3 and anti-testosterone antibody. The two predominant proteins that found in Bali cattle (*Bos sondaicus/javanicus*) were 100% identical to those of complement C3 and anti-testosterone antibody of *Bos Taurus* cattle. As far as our concerned these findings are novel in Bali cattle follicles. A further study needs to be carried out to elucidate the role of the proteins in the female Bali cattle reproductive system.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTION

BR contributed in sample collection, and together with SND designed and carried out the experiment. SND also supervised the research work, data collection, proteomic analysis as well as drafted the manuscript. ASD contributed in supervising on biology reproduction system. All the authors read and approved the manuscript before submission.

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