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Proteomic Analysis of Pesisir Bull Sperm in Different Age Groups for the Identification of Reproductive Function Proteins

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ABSTRACT

Pesisir cattle are native to Indonesia and originate from West Sumatra. It is known for its high environmental adaptability and can be further developed using a proteomic approach. The proteomic approach for testing the fertility of Pesisir bulls is an important factor in determining a potentially superior breed. Proteomic studies of reproduction in Pesisir bulls have not been widely conducted. This study aimed to identify and characterize functional sperm proteins in Pesisir bulls that are associated with reproductive processes. Semen samples were collected from 12 pesisir bulls aged 2–4 years and grouped into three age categories. Protein profile analysis was performed using polyacrylamide gel electrophoresis with 1D-SDS-PAGE, followed by proteolytic digestion of the proteins in the gel and protein identification using LC-MS/MS analysis. Protein functions were predicted based on analysis of biological annotations generated using UniProt, Venny, PANTHER, and STRING. Based on the evaluation of fresh semen, sperm motility in Pesisir bulls was found to increase with age. Proteomic analysis successfully identified 334 proteins in the sperm of Pesisir bulls. Among these, two proteins, ZPBP and SPACA3, were identified as involved in reproductive and fertilisation processes. Functionally, both proteins play crucial roles in acrosomal events during fertilisation. Gene Ontology analysis showed that most proteins in sperm are involved in various biological processes, including cellular activities, metabolic processes, and molecular functions related to catalytic activities. In conclusion, SPACA3 and ZPBP proteins were identified as potentially involved in reproductive processes and may serve as fertility markers in Pesisir bulls.

Keywords: Pesisir bulls; proteomic; markers; ZPBP; SPACA3

INTRODUCTION

The Pesisir cattle is one of Indonesia's indigenous breeds officially recognized as a genetic resource, as stated in the Minister of Agriculture Decree Number: 2908/KPTS/OT.140/6/2011. This pesisir breed is commonly raised in communities living in the coastal regions of West Sumatra. Without reducing performance, Pesisir cattle have advantages such as disease resistance, tropical climate adaptability, and low-quality feed tolerance. The rearing system uses the traditional semi-intensive method, in which cattle are released in the morning and penned in the afternoon, with the main feed coming from forages that are widely obtained from community gardens (Wahyuni & Dewi, 2018).

Pesisir cattle exhibit high environmental adaptability and can maintain optimal production even under suboptimal conditions. These cattle hold significant potential as superior genetic resources for advancing national livestock development. Genetic improvement efforts are essential to enhance both productivity and reproductive performance. However, the breeding soundness examination (BSE) method has limitations, particularly in detecting submicroscopic defects that may impair fertility. Therefore, molecular approaches such as proteomics offer more profound insight into functional sperm quality and fertility potential (Klein *et al*., 2022).

Increasing the population and performance of pesisir cattle requires the identification of superior bulls using molecular technology approaches that

focus on reproductive markers. The application of omics technology, particularly proteomics, represents a significant advancement in bull selection by enabling the identification of proteins that regulate fertility (Sanches *et al*., 2024). Proteomic analysis offers a comprehensive understanding of male reproductive biology by detecting specific proteins and their associated molecular mechanisms (Selvam *et al*., 2020; Kaltsas *et al*., 2024; Rusdin *et al*., 2020). This approach has proven reliable for identifying fertility-related biomarkers and protein markers linked to reproductive efficiency (Agarwal *et al*., 2020; Rosyada *et al*., 2023).

Several fertility-associated proteins have been identified in bull semen, including SPACA1, PEBP1, and BEBP4 in Simmental cattle (Satrio et al., 2024), and TEXT101, BSP1, BSP3, SPADH2, and PRSS55 in Balinese cattle, which are linked to sperm motility (Diansyah *et al*., 2025). These findings highlight the potential of semen proteomics as a predictive tool for male fertility (Gacem *et al*., 2023) and for improving sire selection through sperm protein-based markers (Kusumawati *et al*., 2023).

In light of current scientific literature, a comprehensive proteomic profiling of Pesisir bull sperm has yet to be reported. This highlights a critical gap in the molecular characterization of this indigenous breed, particularly concerning reproductive traits essential for marker-assisted selection. Given the limited molecular information, a detailed understanding of sperm protein composition is vital to support fertility-based selection strategies. Accordingly, this study aims to identify and characterize functional sperm proteins involved in the reproductive processes of Pesisir bulls, thereby establishing a molecular foundation for more accurate and effective breeding programs.

MATERIALS AND METHODS

Animals and Experimental Design

This study involved 12 Pesisir bulls aged between less than 2 and 4 years, managed under standard husbandry practices at BPTU-HPT Padang Mangatas. Fresh semen samples were collected from each individual and grouped into three age categories: <2 years (PS<2), 2–3 years (PS3), and 4 years (PS4). The research protocol was approved by the Animal Research Ethics Committee, Faculty of Veterinary Medicine and Biomedical Sciences, IPB University (Approval No. 221/KEH/SKE/VII/2024). A schematic overview of the sperm protein identification workflow is illustrated in Figure 1.

Extraction of Sperm Protein and 1D-SDS Page Analysis

The semen was centrifuged at 6500 rpm, 4 **°C for** 30 minutes to separate the sperm from the seminal plasma. The sperm pellets were extracted using PRO-PREPTM Protein Extraction Solution (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. PRO-PREP solution as much as 400 µL was added to the sperm pellet and incubated at −20 °C for 20 minutes. The mixture was centrifuged at 13,000 rpm (4 °C) for 5 minutes, and the supernatant was transferred into a sterile tube. The total soluble protein concentration of the seminal plasma and sperm samples was measured before SDS-PAGE analysis using the bicinchoninic acid (BCA) protein assay method (Thermo Scientific™, United States).

SDS-Page analysis was performed to determine the protein profile based on molecular weight, depicted in the form of bands on the gel. Protein separation was performed using SurePAGE™, Bis-Tris, 10 × 8 cm, 12 wells, 4%–20% gradient gel (M00656; GenScript) (SurePAGE, GenScript Biotech Corp., Hong Kong), with running buffer Tris-MOPS-SDS (M00138; GenScript). Electrophoresis was performed at a voltage of 200 V and a current of 100–120 mA for 50 minutes. The gel was then stained using Coomassie Brilliant Blue stain (R-250; Bio-Rad, United States). The Broad Multi Color Pre-Stained Protein Standard (M00624; GenScript) was used as a marker with a molecular weight range of ~5–270 kDa (Maulana *et al*., 2025).

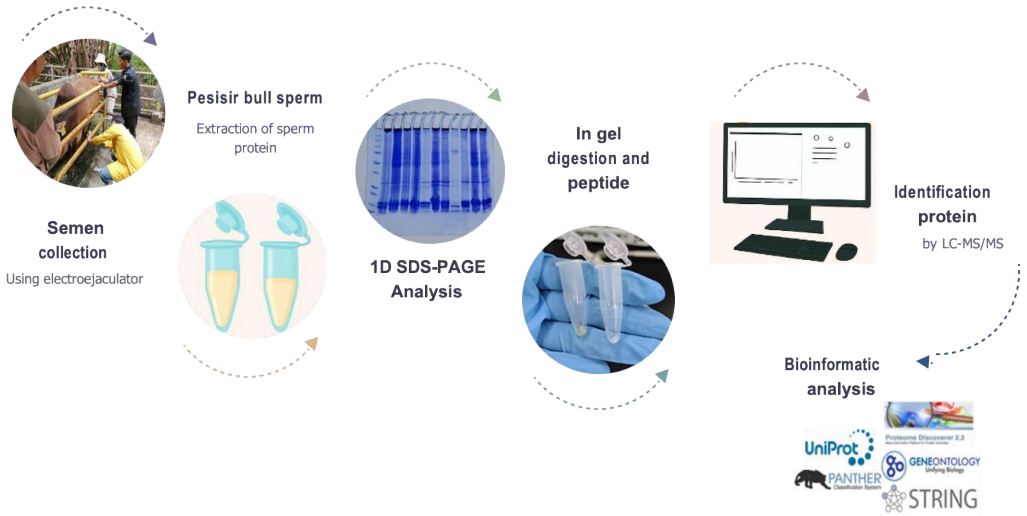


Figure 1. Workflow diagram illustrating the steps of proteomic analysis in sperm from Pesisir bulls

In Gel Tryptic Digestion and LC–MS/MS Analysis

Protein bands were excised from acrylamide gels into 1 × 1 mm pieces and transferred to sterile microcentrifuge tubes. Destaining was performed twice using 200 µL of solution containing 80 mg ammonium bicarbonate in 20 mL acetonitrile (ACN) and 20 mL ultrapure water, and was incubated at 37 °C for 30 minutes with gentle agitation. Reduction of cysteine residues was carried out with 30 µL of TCEP solution (3.3 µL TCEP in digestion buffer) at 60 °C for 10 minutes, followed by alkylation using 30 µL iodoacetamide solution at room temperature in the dark for 1 hour. The gel pieces were washed twice with destaining solution at 37 °C for 15 minutes. ACN was added to dehydrate the gel pieces for 15 minutes, followed by air drying for 5–10 minutes for digestion. Then, 10 µL of trypsin solution (10 ng/µL) was added, incubated for 15 minutes, and continued with 25 µL of digestion buffer. Digestion proceeded for 4 hours at 37 °C or overnight at 30 °C with agitation. The reaction was stopped with 10 µL of 1% TFA or formic acid for 5 minutes.

Peptide purification was conducted using Pierce™ C18 Spin Columns. The resin was activated with 200 µL of 50% ACN, followed by equilibration with 200 µL of 0.5% TFA in 5% ACN. A total of 150 µL of the digested sample was loaded and centrifuged at 1500 g for 5 minutes. After washing with equilibration buffer, peptides were eluted with 20 µL of 70% ACN and dried using a SpeedVac. Dried peptides were reconstituted in 50 µL of 2% ACN with 0.1% formic acid, centrifuged at 12,000 rpm for 10 minutes, and 2.5 µL was injected into a Nano LC Ultimate 3000 system coupled with Q Exactive Plus Orbitrap HRMS (Thermo Scientific). Separation was performed using a 30 µm × 5 mm trap column (Thermo Scientific™ 164649) and a PepMap RSLC C18 analytical column (75 µm × 15 cm, 3 µm particle size, 100 Å pore size) at 300 nL/min.

The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in ACN). Gradient elution was set as follows: 2%–35% B over 27 min, 35%–99% B over 10 min, 99% B for 15 min, and re-equilibration at 2% B for 30 min. Mass spectra were acquired using an LTQ-Orbitrap in the 200–2000 m/z range (Thermo Scientific, Bremen, Germany).

Data Processing and Bioinformatics

Mass spectra were processed using Proteome Discoverer 2.2 (Thermo Fisher Scientific) with Sequest HT against the Bos taurus protein database (UniProt). Only proteins with ≥2 unique peptides, HT score >0, and within 10 ppm mass error were considered valid. Common contaminants such as keratin were excluded. Functional categorization of proteins was performed using PANTHER (http://pantherdb.org), while Venn diagrams were generated using Venny 2.1.0. STRING v12.0 (https://string-db.org/) was used for mapping protein interactions and biological functions, including cellular localization and molecular activities.

RESULTS

Fresh Semen Quality of Pesisir Bulls

The evaluation of fresh semen quality from 12 Pesisir bull samples across various age groups indicated increased sperm parameters with age. Progressive motility increased with age, recorded at 69.54 ± 25.19% in bulls aged <2 years, 85.05 ± 3.87% in those aged 2–3 years, and peaking at 91.53 ± 4.06% in 4-year-old bulls. A similar trend was observed in sperm viability, which rose from 64.56 ± 16.08% (<2 years) to 78.23 ± 11.29% (2–3 years), with a slight decline to 77.63 ± 1.53% in the 4-year age group. Conversely, sperm abnormalities decreased, from 19.03 ± 1.92% in bulls aged <2 years to 18.61 ± 1.73% (2–3 years), and the lowest at 16.23 ± 2.71% in 4-year-old bulls (Table 1). These findings suggest that sexual maturity and optimal semen quality in Pesisir bulls are generally achieved after two years.

Sperm protein distribution in pesisir bulls: molecular weight, isoelectric point, HT Score, and unique peptides. The proteins identified in pesisir bull sperm had a distribution range of 10–150 kDa. Proteins with molecular weights of 21–40 kDa and 41–60 kDa were the most dominant, with even distribution across all age groups. Figure 2 illustrates the proteomic distribution of the Pesisir bull sperm proteins categorized by molecular weight, isoelectric point (pI), unique peptide count, and HT Score. The identified proteins have a molecular weight range of 10-150 kDa, with the highest in the 21-40 kDa range, and the lowest in the 101-150 kDa range, which is spread across all age groups. The pI distribution ranged from 4.01-12.0, the highest being 8.01-9.0 and the lowest being 4.01-5.0 across all age groups. Based on the number of unique peptides, the total number of unique peptides was 2–10, the highest was 2, and the lowest was 8. The total HT Score varied between age groups, with 73 proteins with a value of 4.13-116.77 at <2 years old, 167 proteins with a value of 3.97-170.60 at 2-3 years old, and 94 proteins with a value of 4.27-112.83 at 4 years old.

Protein Distribution and Venny Analysis

A total of 647 sperm proteins were identified from 12 pesisir bulls grouped into three age groups (Table 2). A total of 334 proteins were identified, whereas the rest were not analyzed further. The protein distribution between the age groups was analyzed using Venn diagrams (Figure 3). The results showed that 8 sperm-specific proteins (4.3%) were only expressed in pesisir

Table 1. Fresh semen quality of Pesisir bulls divided into three age groups

Fresh semen quality	Age of Pesisir bulls		
	<2 year	2-3 years	4 year
	Mean±SD	Mean±SD	Mean±SD
Motility (%)	69.54 ± 25.19	85.05 ± 3.87	91.53 ± 4.06
Viability (%)	64.56 ± 16.08	78.23 ± 11.29	77.63 ± 1.53
Abnormality (%)	19.03 ± 1.92	18.61 ± 1.73	16.23 ± 2.71

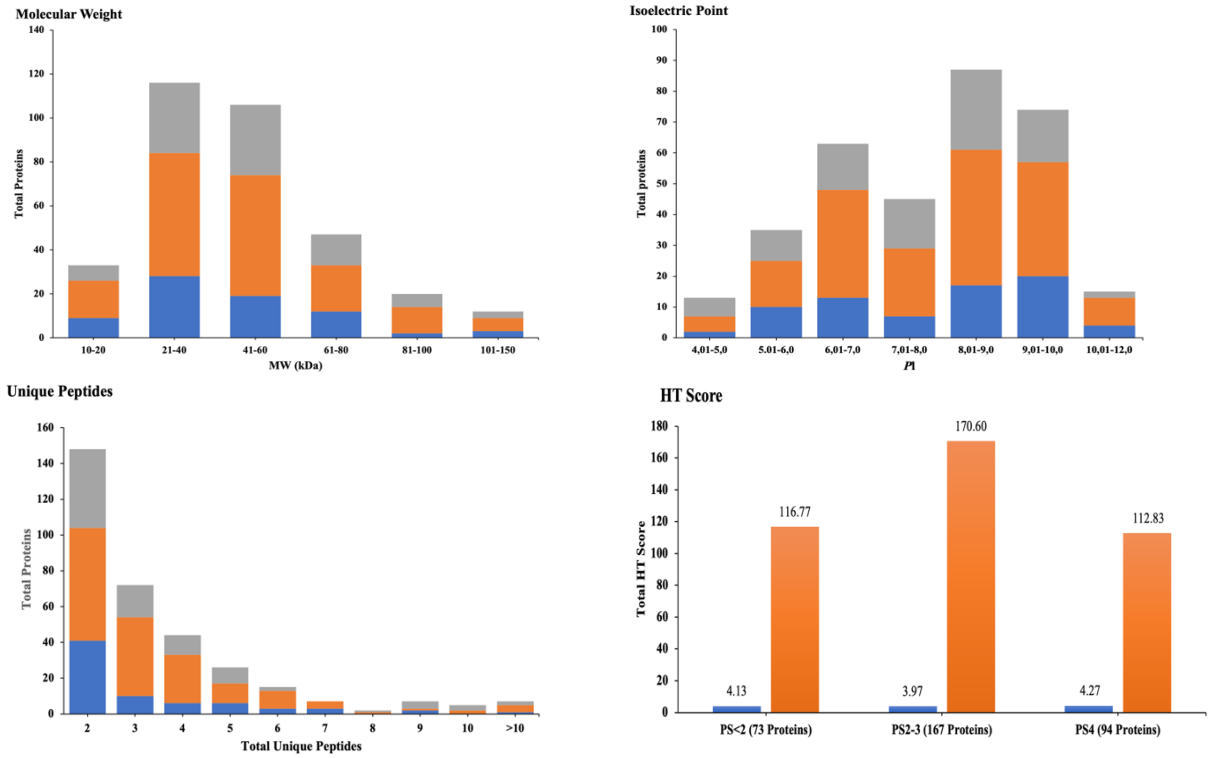


Figure 2. Proteomic distribution based on molecular weight, isoelectric point, unique peptides, and sequest high throughput (HT) score in the sperm protein of Pesisir bulls. Fresh semen samples were collected from Pesisir bulls grouped into three age categories: 4 years (PS4), ■; 2–3 years (PS2-3), ■; <2 years (PS<2), ■.

Table 2. Identification of sperm proteins in Pesisir bulls using LC-MS/MS analysis

Protein	Total protein	Age of Pesisir bulls (Years)		
		PS<2	PS2-3	PS4
Protein discovery 2.2	647	161	295	191
Selection result	334	73	167	94

Note: Sperm protein (PS); PS<2: Pesisir bulls aged <2 years, PS2-3: Pesisir bulls aged 2–3 years, PS4: Pesisir bulls aged 4 years.

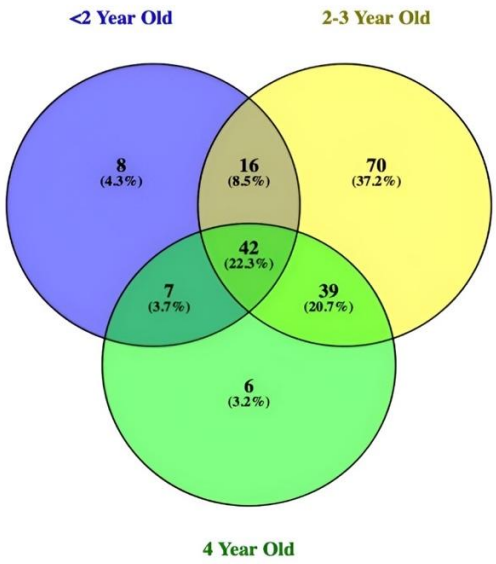


Figure 3. Protein-specific expression in Pesisir bull sperm according to age groups: (<2 years, 2-3 years, and 4 years old).

bulls less than 2 years old, 70 proteins (37.2%) were only expressed at 2-3 years old, and 6 proteins (3.2%) were only expressed at 4 years of age. A total of 42 proteins (22.3%) were consistently represented across all three age groups. Meanwhile, 16 proteins (8.5%), 39 proteins (20.7%), and 7 proteins (3.7%) were expressed in the two different age groups.

Protein Classification Based on Gene Ontology (GO) Analysis

GO classification of Pesisir bull sperm proteins revealed their involvement in several key biological processes across all age groups. The predominant GO categories were Cellular Process (GO:0009987), Metabolic Process (GO:0008152), Biological Regulation (GO:0065007), Response to Stimulus (GO:0050896), Developmental Process (GO:0032502), Multicellular Organismal Process (GO:0032501), Immune System Process (GO:0002376), and interspecific interaction (GO:0044419). Cellular and metabolic processes were the most dominant functional categories across all the age groups. Based on the classification of proteins related to reproductive function, reproduction (GO:0022414),

and reproductive process (GO:0000003), two specific proteins were identified as being expressed in the 2–3 year and 4 year age groups, but were not expressed in bulls under 2 years of age (Figure 4A).

Analysis of Pesisir bull sperm protein revealed consistent molecular functions across all age groups, encompassing roles such as catalytic activity (GO:0003824), binding (GO:0005488), structural molecule activity (GO:0005198), and antioxidant activity (GO:0016209). Meanwhile, molecular functions, such as transporter activity (GO:0005215) and molecular function regulator activity (GO:0098772), were only found in the 2-3 year age group and not in other age groups. Among all categories, catalytic activity and binding molecular functions were highest across all age groups (Figure 4B).

According to the cellular component classification, sperm proteins of Pesisir bulls were assigned to the categories of cellular anatomical entity (GO:0110165) and protein-containing complex (GO:0032991), with both classifications consistently observed across all age groups. Most proteins associated with cellular components were abundant in bulls aged 2–3. Based on the results, the GO analysis classification, which includes biological processes, molecular functions, and cellular components, shows variations in the number and types of sperm proteins among different age groups. This indicates that differences in protein expression may play a role in influencing reproductive function in Pesisir bulls (Figure 4C).

Specific proteins sperm in the reproductive process of Pesisir bulls. Panther software analysis identified two specific proteins associated with reproduction (GO:0022414) and reproductive processes (GO:0000003). Expression of these reproduction-related proteins was observed in the 2–3 and 4-year age groups: zona pellucida binding protein (ZPBP) and ID protein F1N369. Furthermore, acrosome-associated specific protein 3 (SPACA3), ID Protein A6QQ77, was found only in the 4-year-old group. In contrast, the <2-year-old group did not show the expression of specific proteins related to reproductive function, as shown in Table 3.

Interaction of Protein-Specific Sperm in the Reproduction of Pesisir Bulls

The analysis showed that the low false discovery rate (FDR) value in STRING indicated a high significance level for protein interactions. SPACA3 protein is

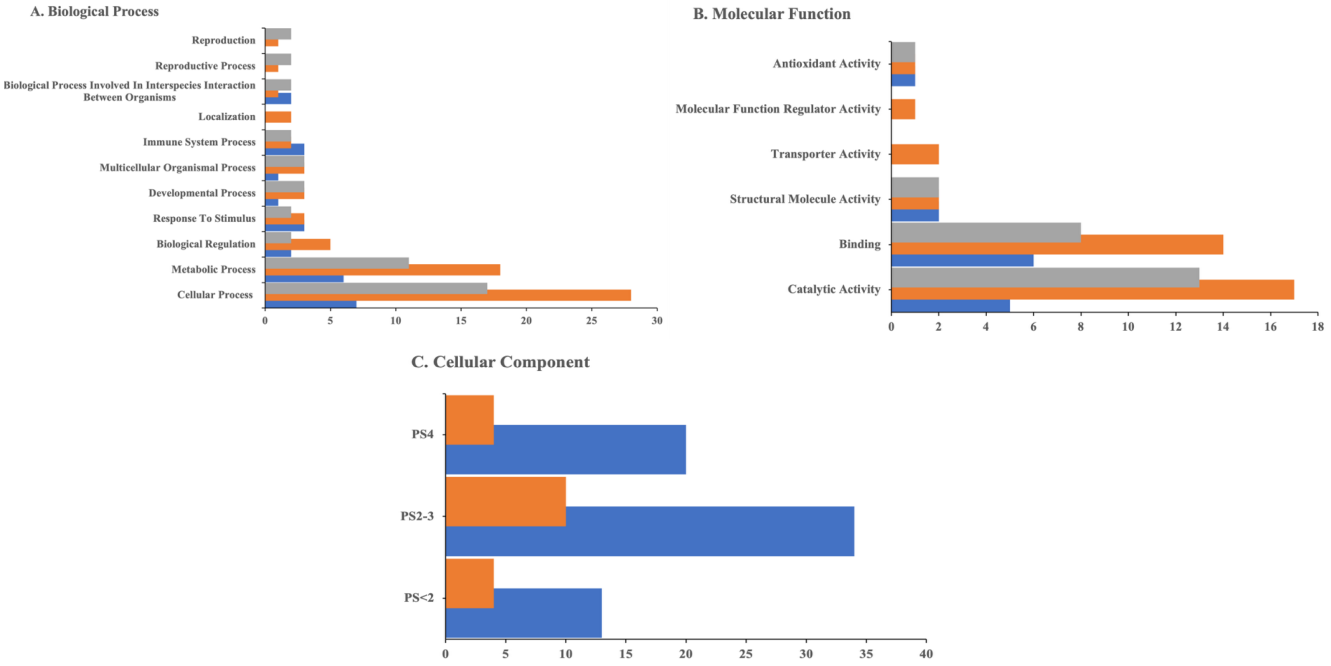


Figure 4. Classification of proteins based on gene ontology (GO) analysis through biological processes (a), molecular functions (b), and cellular components (c) in Pesisir bulls sperm proteins. Fresh semen samples collected from Pesisir bulls grouped into three age categories: 4 years (PS4), ■; 2–3 years (PS2-3), ■; <2 years (PS<2), ■. Protein-containing complex, ■; Cellular anatomical entity, ■.

Table 3. Age-specific sperm protein expression associated with reproductive function in Pesisir bulls (PANTHER)

Protein ID	Gen name	Function
2-3 and 4-year-old		BP/MF/CC
F1N369	ZPBP	Acrosomal vesicles help sperm recognise and penetrate the zona pellucida (ZP), and play a role in fertilization.
4-year-old		
A6QQ77	SPACA3	A membrane protein on the surface of sperm involved in the process of adhesion and plasma membrane fusion between sperm and egg during fertilization.

involved in various biological processes related to reproduction, such as multicellular organism reproduction (GO:0032504), sexual reproduction (GO:0019953), reproductive processes (GO:0022414), cellular processes in reproduction (GO:0022412), and spermatogenesis (GO:0007283). In addition, SPACA3 is associated with cellular components, such as acrosomal vesicles (GO:0001669), acrosomal membrane (GO:0002080), and inner acrosomal membrane (GO:0002079). ZPBP proteins are involved in several key biological processes, including sexual reproduction (GO:0019953), spermatid development (GO:0007286), and acrosome formation (GO:0001675). Meanwhile, the protein function related to the cellular component was the acrosomal vesicle component (GO:0001669), as shown in Table 4.

The STRING database revealed the presence of reproduction-associated proteins in Pesisir bull sperm, specifically sperm acrosome membrane-associated protein 3 (SPACA3) and zona pellucida binding protein (ZPBP). SPACA3 was uniquely identified in bulls aged 4 years, while ZPBP was detected in the 2–3 and 4-year age groups, but was absent in bulls aged less than 2 years. The analysis also demonstrated that SPACA3 interacts directly with several proteins, including IZUMO1, ACRBP, PRM3, TSGA10, TMEM190, and SPACA1, which are involved in zona pellucida penetration and sperm-oocyte membrane fusion (Figure 4A). Additionally, ZPBP directly interacted with ACRBP, SPACA1, ROPN1, and SPA17 (Figure 4B), suggesting that both SPACA3 and ZPBP play critical roles in regulating spermatogenesis and the acrosome reaction essential for successful fertilization.

DISCUSSION

The protein distribution results showed that the proteins found in Pesisir bull sperm had molecular weights between 21 and 150 kDa. Proteins with the highest MW were found in the 21-60 kDa range. Sperm proteins have isoelectric points between 4 and 8 pI. The highest dominance was in the range of 8.01-9.0 pI. In addition, the protein contains some unique peptides between 2 and 10. The HT scores varied from 4.13 to 170.60 (Figure 2). Gene Ontology analysis of protein classification revealed that proteins involved in metabolic processes were most prevalent, particularly within the molecular weight range of 21–60 kDa. This finding was consistent with the report by Baharun *et al*. (2023). The detection of proteins with molecular weights between 50–65 kDa in semen plasma and sperm suggests their involvement in catalytic activities, likely in energy metabolism regulation, which is essential for sperm functionality.

A total of 334 sperm proteins were identified through proteomic analysis of bulls from the three different age groups (Table 2). Specifically, 73 proteins were found in bulls less than 2 years old, 167 in 2-3-year-old bulls, and 94 in bulls 4 years old (Figure 3). Various studies on livestock species have revealed the diversity of sperm protein profiles. Satrio *et al*. (2024) reported 127 sperm protein profiles in Simmental bulls, classified by age group. Diansyah *et al.* (2023) identified 196 protein profiles in Balinese bulls, whereas Rosyada *et al.* (2023) found 15 proteins in Madura cattle sperm that play a role in sperm function. Kumaresan *et al.* (2023) found a much larger number and identified 1,305 protein profiles related to reproductive function in buffalo sperm. Among the 334 identified proteins, cellular and metabolic processes were the most common. In addition, immune system processes were identified.

This study successfully identified proteins in the sperm of pesisir bulls of different age groups. GO classification based on gene ontology showed that proteins related to cellular and metabolic processes

Table 4. STRING analysis of sperm proteins specifically associated with the reproductive function of Pesisir bulls

Protein (Accession)	Go-term	Protein function	False discovery rate	Associated proteins
4-year-old				
SPACA3 (A6QQ77)	GO:0032504	Multicellular organism reproduction (BP)	0.0018	SPACA3, IZUMO1, ACRBP, SPACA1, PRM3, TSGA10
	GO:0019953	Sexual reproduction(BP)	0.0018	SPACA3, IZUMO1, ACRBP, SPACA1, PRM3, TSGA10
	GO:0022414	Reproductive process (BP)	0.0183	SPACA3, IZUMO1, ACRBP, SPACA1, PRM3, TSGA10
	GO:0022412	Cellular process in reproduction (BP)	0.0357	SPACA3, IZUMO1, ACRBP, SPACA1
	GO:0007283	Spermatogenesis (BP)	0.0498	ACRBP, SPACA1, PRM3, TSGA10
	GO:0001669	Acrosomal vesicle (CC)	7.60e-07	SPACA3, SPACA1, IZUMO1, TMEM190, ACRBP
	GO:0002080	Acrosomal membrane (CC)	7.60e-07	SPACA3, SPACA1, IZUMO1,TMEM190.
	GO:0002079	Inner acrosomal membrane (CC)	0.0019	SPACA1, TMEM190
2-3 and 4-year-old				
ZPBP (F1N369)	GO:0019953	Sexual reproduction (BP)	0.0140	ZPBP, ACRBP, SPACA1, ROPN1, SPA17
	GO:0007286	Spermatid development (BP)	0.0047	ZPBP, ACRBP, SPACA1, ROPN1
	GO:0001675	Acrosome assembly (BP)	0.0021	ZPBP, ACRBP, SPACA1
	GO:0001669	Acrosomal vesicle (CC)	0.0223	ZPBP, ACRBP, SPACA1

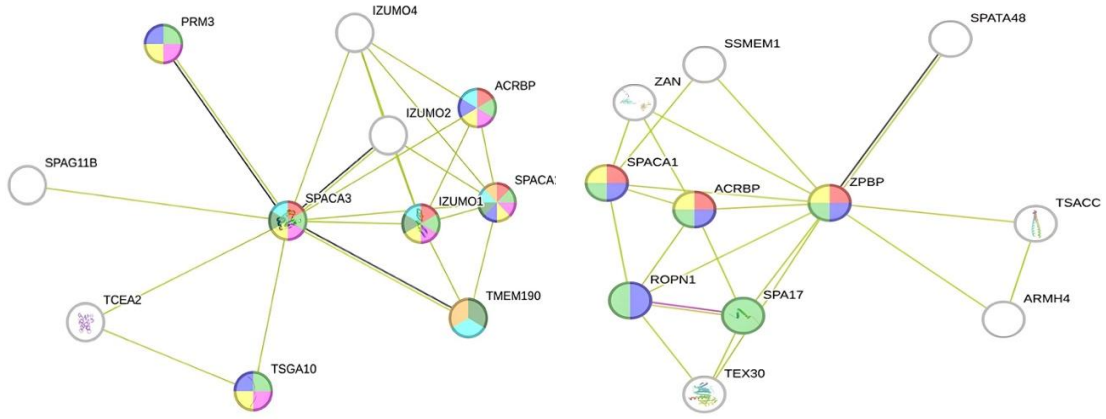
were found in all age groups, suggesting an essential role in sperm physiological activity (Figure 4A). This study aligns with the findings of Satrio *et al*. (2024), which also showed that Simmental bulls from all age groups express proteins essential for sperm metabolic function, including processes such as capacitation, acrosome reaction, and fertilization. This metabolism is essential for the production of energy, which supports sperm motility. Energy comes from the hydrolysis of ATP, which is produced through the glycolysis pathway and tricarboxylic acid cycle, as well as from the OXPHOS and glycolysis pathways (Gunawan *et al*., 2018; Kumaresan *et al*., 2023). Sperm metabolic processes and molecular catalytic activity are the most dominant biological functions found in the sperm proteins of pesisir bulls, which is in line with the finding of Sanchez *et al*. (2022) that sperm motility, which is highly dependent on energy availability, is a major predictor of successful egg fertilization.

Based on the biological process classification using PANTHER analysis, the proteins ZPBP and SPACA3 were expressed in the sperm of Pesisir bulls aged 2 to 4 years. Their absence in bulls under 2 years of age may be attributed to the onset of sexual maturity. This pattern is consistent with previous reports in Simmental and Bali bulls (Satrio *et al*., 2024; Diansyah *et al*., 2025; Gunawan *et al*., 2011), which indicate that proteomic expression in male cattle is age-dependent, with proteins involved in acrosome assembly and spermatid development, such as members of the SPACA family, beginning to emerge between 2 and 4 years of age. These findings suggest that the expression of ZPBP and SPACA3 is developmentally regulated and closely associated with reproductive maturation.

This is further supported by semen quality data from Pesisir bulls, which demonstrated increased sperm motility with age. Functionally, ZPBP and SPACA3 play key roles in sperm binding to the zona pellucida during fertilization. In line with this, Diansyah *et al.* (2025) reported a significant correlation between ZPBP levels in the seminal plasma of Bali bulls and sperm quality, suggesting its potential as a fertility biomarker. Similarly, Maulana *et al*. (2025) identified ZPBP as a reproduction-specific protein in the sperm proteome of Toraja buffalo. Moreover, Lin *et al.* (2007) emphasized the critical function of ZPBP in sperm-egg interaction, acrosome biogenesis, and morphological development during spermiogenesis.

Based on the results of STRING analysis, ZPBP proteins in pesisir bull sperm are involved in various critical biological processes, including sexual reproductive proteins, spermatid development, acrosome formation, and cellular components that play a role in acrosome vesicle formation. This supports the report of Song *et al.* (2010) that during fertilization, sperm bind specifically to the extracellular matrix of the zona pellucida (ZP), which then triggers the acrosome reaction during the process of ZP penetration and successful fertilization. Furthermore, the protein interaction analysis results showed that ZPBP directly interacted with ACRBP, SPACA1, ROPN1, and SPA17 proteins, and these proteins work synergistically with each other to support fertility (Figure 5B). Jin *et al.* (2011) showed the interaction of ZPBP proteins in sperm that maintains intact acrosomes during capacitation to reach the cumulus oophorus. Then, research by Corda *et al.* (2024) showed a positive correlation between ZPBP expression and function in maturation, motility, capacitation, and acrosome reaction, which has the potential to be used as a biomarker for assessing semen quality and fertility.

STRING analysis revealed that SPACA3 protein directly interacts with IZUMO1, ACRBP, PRM3, TSGA10, TMEM190, and SPACA1 (Figure 5A), synergistically supporting successful fertilization. The interaction between IZUMO1 and TMEM190 plays a role in mouse sperm capacitation (Falco *et al*., 2022) and ACRBP in boars (Kato *et al*., 2021). Consistent with this, the results of cellular component function annotation from STRING analysis indicated that SPACA3 is associated with cellular structures such as acrosome vesicles, outer acrosome membranes, and inner acrosome membranes. Leung *et al.* (2023) reinforced these findings by showing that human sperm that could attach to the ZP had a higher SPACA3 expression and exhibited a more optimal level of capacitation.



(a) (b)

Figure 5. Protein interaction of SPACA3 in 4-year-old Pesisir bulls sperm (a) and ZPBP in 2-3-year-old and 4-year-old Pesisir bulls sperm (b), associated with reproductive function (String).

Regarding the acrosome reaction, Nagdas *et al.* (2017) explained that the formation of a fenestrated hybrid complex on the sperm plasma membrane facilitates the release of hydrolytic enzymes essential for ZP penetration.

Specifically regarding the function of the SPACA protein family, Harayama *et al.* (2017) reported that SPACA1 is localized to the main segment of the sperm acrosome in Japanese Black bulls. This protein was positively correlated with successful fertilization through artificial insemination. Furthermore, Zhou *et al*. (2024) identified SPACA1 as an essential protein for supporting sperm function, especially for maintaining the structure of the acrosome and sperm head. SPACA1 deficiency is associated with abnormal sperm head morphology and acrosome formation. Meanwhile, Agil *et al.* (2025) found that SPACA1 levels in Balinese bull semen were low, which was associated with decreased protein concentration and overall semen quality. A decrease in SPACA1 levels was interpreted as an indication of decreased fertility in Balinese cattle. Furthermore, Zheng *et al*. (2015) described SPACA3 as a lysozyme-like protein predominantly expressed in mammalian testes, localized in sperm acrosomes, and exhibiting a strong affinity for egg plasma membrane receptors, with additional roles in early embryonic development. On the other hand, Korfanty *et al.* (2012) characterized SPACA7 as an acrosome-specific protein released during the acrosome reaction that has an essential role in fertilization. In line with these findings, this study identified two specific proteins, SPACA3 and ZPBP, which have essential roles in the reproductive process and have the potential to become sperm fertility markers in pesisir bulls.

CONCLUSION

SPACA3 and ZPBP proteins were identified as potentially involved in reproductive processes and may serve as fertility markers in Pesisir bulls. Their age-dependent expression suggests roles in sexual maturity, offering molecular insight for fertility assessment. Further validation is needed to confirm their functional relevance.

CONFLICT OF INTEREST

C. Sumantri and A. Gunawan serve as editors of the Tropical Animal Science Journal but have no role in the decision to publish this article. The authors have no conflicts of interest related to the manuscript's content.

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