

Characterization of Tyrosine Kinase Protein in Spermatozoa Plasma Membrane of Merino Sheep

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Abstract : This study aims to identify and isolate tyrosine kinase derived from spermatozoa plasma membrane of Merino sheep. The procedure was done by taking a sample of Merino sheep semen which was further centrifuged to obtain the solid part (spermatozoa). Then, the identification of the Merino sheep's spermatozoa sample was conducted to obtain crude tyrosine kinase protein using SDS PAGE and electroelution was then performed to obtain tyrosine kinase isolates. The conclusions of this study are as follows: Tyrosine kinases can be identified from the spermatozoa plasma membrane of Merino sheep using SDS-PAGE with molecular weight of 95.55 kDa. 2). Tyrosine kinase isolation from spermatozoa plasma membrane of Merino sheep can be done by using electroelution. The mean level of tyrosine kinase isolates is 233.2 ug/ml.

Key words: tyrosine kinase isolates, spermatozoa plasma membrane, Merino Sheep.

Introduction

In supporting Artificial Insemination (AI) program, we require the provision of semen sufficient in quality and quantity in the form of fresh semen or frozen semen. Fresh semen quality declines faster than frozen semen though stored in wither in diluent medium or without diluent medium. Semen can be stored in cold conditions (in a refrigerator) or in freezing conditions (in liquid nitrogen). Cooled semen has relatively short durability, while that stored in frozen state allows long term use¹.

The increase of sheep population in Indonesia with phenotype and genotype advantages can be attempted by artificial insemination using superior male spermatozoa. Merino sheep is one of the sheeps with advantages of producing meat and wool². Sheep semen has a low volume but high concentrations³. In freezing process, sheep spermatozoa have lower viability than cow spermatozoa⁴.

Freezing is a temporary cessation of cell metabolism processes without shutting down cell function. The metabolic process may continue after the hold is removed¹. In freezing process, semen quality is declining, causing sperm cell death by more than 30%⁵. Changes in several characteristics of plasma membrane as a result of freezing process are, among others, membrane reorganization, increased calcium level, increased reactive oxygen species (ROS) and decreased ability of the sperm to fertilize. Damage to the cell membrane integrity affects the function of the cell membrane components⁶.

Tyrosine kinase (TK) is one of the spermatozoa plasma membrane protein that serves as the main fusion mediator of spermatozoa with zona pellucida 3 (ZP3)⁷. Tyrosine kinase is one protein molecule that is present in spermatozoa plasma membrane and serves introduction to ZP3 and plays a role in signal transduction that may result in autophosphorylation of tyrosine residues⁸. Some spermatozoa membrane proteins in mice

have the ability to bind proteins of zona pellucida (ZP)⁹. Ovum has molecular weights of 95, 63, 51 and 14-18 kDa. However, those having the most potential autophosphorylation activity are proteins with molecular weight of 95 kDa. Proteins with a molecular weight of 95 kDa include into the protein tyrosine kinase. Tyrosine kinases include in the group of proteins that one of its functions is a covalent bond stabilization of membrane proteins constituent, so that when it is added within a diluent medium for frozen semen, it will be able to prevent the rupture of spermatozoa membrane covalent bonds¹⁰.

This study aimed to determine whether tyrosine kinase can be identified from the plasma membrane of Merino sheep's spermatozoa using SDS-PAGE and whether the results of identification of tyrosine kinase of Merino sheep's spermatozoa plasma membrane can be isolated using electroelution techniques.

Materials and Methods

This study was an experimental laboratory study to identify and isolate tyrosine kinase derived from spermatozoa plasma membrane of Merino sheep. The study was conducted at Taman Ternak Pendidikan and Frozen Semen Unit, Faculty of Veterinary Medicine, Universitas Airlangga, at District Kedamean Gresik, East Java Indonesia. This was a descriptive study presented in figures and tables.

Procedure

Crude protein separation from spermatozoa plasma membrane of Merino sheep

Semen samples were collected in a test tube. After macroscopic (volume, color, consistency and pH) and microscopic (motility, mass movements and the percentage of live sperm) examinations, samples were centrifuged at 2000 rpm for 30 minutes to separate the pellet (spermatozoa) with seminal plasma. Then, the pellets were coupled with 1 ml BO-cafein medium, centrifuged at 3000 rpm for 10 minutes. The supernatant was removed, the precipitate was added with PBS-tween 5 times of the volume, added with Phenylmethanesulfonyl fluoride (PMSF) as protease enzyme inhibitor of as much as 5 times of the volume, and then vortexed for 10 minutes, followed by sonication for 20 minutes, and centrifugation at 6000 rpm for 10 minutes. The precipitate was removed, and the supernatant was coupled with cold ethanol 1:1, stored in refrigerator for 1 hour up to the formation of white spots. Centrifugation was done with a speed of 6000 rpm for 10 minutes, put into the refrigerator again for 5 minutes. Then, the ethanol was removed and dried using tissue and left until the odor of the ethanol is lost. Subsequently, it was coupled with Tris-HCl. The end result was a crude proteins isolate from spermatozoa plasma membrane¹¹.

Characterization and identification of tyrosine kinase by SDS-PAGE

Tyrosine kinase identification using SDS-PAGE aims to describe the feature of some protein bands. The steps were as follows: separating gel was put into the electrophoresis through the wall down to the upper limit. Furthermore, distilled water was added to the upper limit to disperse the separating gel evenly. Then the distilled water was absorbed with tissue paper and stacking gel was added passing through the wall until it was full. Comb (wells printer of the gel) was added and waited until it turned into gel. Furthermore comb removed and cleaned of residual gel with tissue. Produced gel mold was removed and inserted into the chamber, and then soaked in running buffer. Subsequently the mold guiding the sample to be runned was mounted. The research sample, presenting as cow spermatozoa membrane protein isolate as much as 35 ul, was inserted into the mold hole with 200 ul tip. The next step was that the chamber was connected by means of Biorad. Power supply was turned on with the power of 130 V, 30 mA for 1.5 hours. If the gel reaction had reached the bottom, the instrument was switched off and the plate was opened and separated. The results were gel shaped sheet stained with coomassie blue and sackered (shaken) for 30 minutes. Then they were removed and added with destaining liquid and being sackered again for 30 minutes. If liquid was already visibly blue, it was replaced with new destaining fluid, and so on until the liquid became white and the results were several protein bands. The molecular weight would be visible on SDS-PAGE gel mold¹².

Isolation tyrosine kinase by mechanical electroelution

Unstained SDS-PAGE gels were cut along as desired. Each piece of the gel was inserted into selophan bag, then put in glass block containing PBS, then stirred for 24 hours. Every 6 hours the PBS was replaced. To

recognize that the protein has been eluted, the gel pieces were stained with silver staining, no band indicated that the protein has been eluted¹³.

Results and Discussion

Macroscopic and microscopic examination of semen Merino sheep

Macroscopic and microscopic examinations of Merino sheep semen were performed to observe fresh semen characteristics of Merino sheep. Macroscopic examination includes volume, odor, color, viscosity (consistency) and pH, while the microscopic examination covered mass movement, individuals movement, viable spermatozoa as well as spermatozoa abnormalities (Table 1).

Table 1. Fresh Semen Characteristics of Merino sheep

Parameters	Notes
Volume (ml)	1.5
Odor	Typical
Color	White
Konsistensi	Thick
pH	6.7
Mass movement	++
Individual movement	75
Viable spermatozoa (%)	93
Spermatozoa abnormality (%)	8.02

Average results of Merino sheep's fresh semen used in this study had met the requirements referred to standard operating procedures (SOP) of the Directorate General of Agricultural Production in 2007 which states that the macroscopic examination of sheep's fresh semen must have an average volume of 1.5 ml, moderate to high consistency, creamy white color, a distinctive odor of the sheep, and the pH ranges from 6.4. Microscopic examination: minimal mass movement ++, individuals movement 70%, percentage of viable spermatozoa 80% and concentration spermatozoa 800 million/ml.

Identification of tyrosine kinase protein in the plasma membrane spermatozoa of Merino sheep

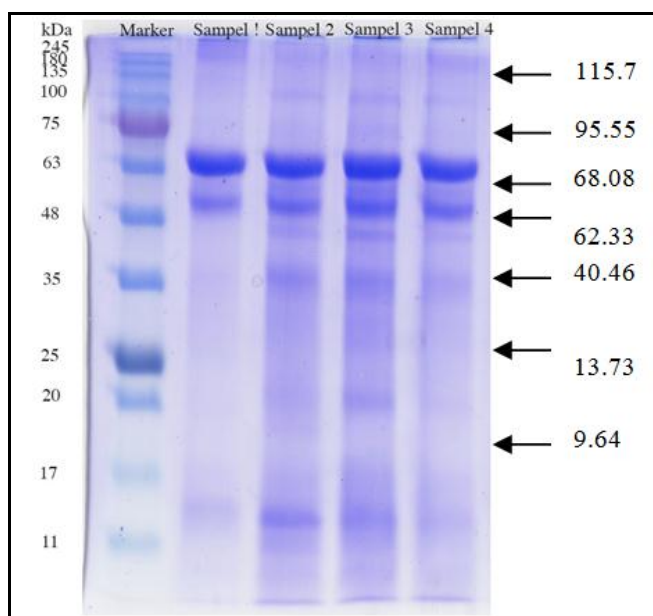


Figure 1. Profile of protein band from plasma membrane spermatozoa of Merino sheep with SDS-PAGE method

Identification of protein profile on plasma membrane spermatozoa of Merino sheep was performed using SDS-PAGE method derived from Merino sheep semen with 8 times taking. Protein bands appeared on the results of SDS-PAGE (Figure 1).

SDS-PAGE electrophoresis method is used to determine the molecular weight of the plasma membrane tyrosine kinase spermatozoa Merino sheep. The working principle of SDS-PAGE method is based on the movement of charged particles through a gel caused by the influence of an electric field¹⁴. The principle of electrophoresis is the velocity of the protein that is affected by molecular weight. When a protein having a molecular weight is small, then the speed will be faster than the protein has a large molecular weight.

Isolation of plasma membrane proteins of Merino sheep spermatozoa using SDS-PAGE showed thirteen (13) proteins, 115.72, 95.55, 79.28, 68.08, 62.33, 53.52, 40.46, 30.13, 21.26, 13.73, 11.06, 9.64 and 8.08 kDa. Molecular weights of protein isolate of Merino sheep's spermatozoa plasma membrane were determined by determining Rf value obtained in the linear regression equation $Y = - + 1.360x 2.180$, correlation curve between Rf (x-axis) with log molecular weight of the standard protein (Y axis) (Figure 2). Furthermore, the thirteen of protein bands were matched with standard protein bands (Marker) whose relative molecular mass (Mr) had been known.

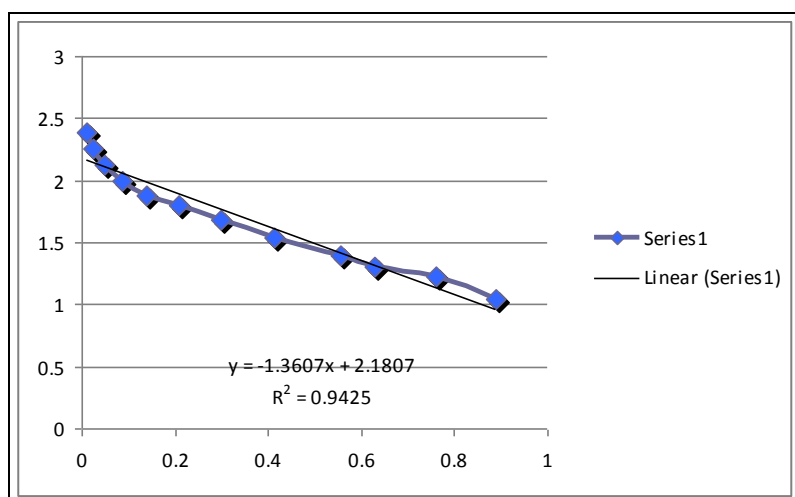


Figure 2. Correlation curve between Rf with standard protein BM log

Various species of mammals have tyrosine kinase protein with a molecular weight very varied. Identification of spermatozoa protein bands in mice based on their molecular weight showed three bands, namely 52, 75 and 95 kDa⁷. One that has the highest immunoreactivity and that able to interact specifically with zona pellucida of the ovum is the tyrosine kinase with molecular weight of 95 kDa. Identified four protein bands in human spermatozoa is a molecular weight of 95 ± 3 kDa, 46 ± 3 kDa, 25 ± 7 kDa and 12 ± 2 kDa, and one that has a high specificity to the zona pellucida egg is protein namely the molecular weight of 95 kDa tyrosine kinase⁹. Proteins that have been identified through SDS-PAGE method still showed some protein bands so we needed more specific test to determine the desired enzyme protein.

Isolation of tyrosine kinase from plasma membrane of sperm Merino sheep using electroelution method

After identification using SDS-PAGE we obtained thirteen protein bands. Tyrosine kinase has a molecular weight of 95.55 kDa. For electroelution method, we cut the unstained gel on protein bands with molecular weight of 95.55 kDa which were subsequently incorporated into a cellophane bag containing PBS media. Electroelution produced liquid containing tyrosine kinase isolates with molecular weight of 95.55 kDa. Measurement of the concentration of tyrosine kinase isolates of plasma membrane from Merino sheep's spermatozoa using NanoDrop methods can be seen in Table 2.

Table 2. Tyrosine kinase isolates concentration of plasma membrane of Merino sheep's spermatozoa

Elution no.	Tyrosine kinase isolate elution ($\mu\text{g/ml}$ elution sample)
1	157.9
2	308.5
Mean	233.2

Tyrosine kinase isolates concentration produced from electroelution of Merino sheep's spermatozoa plasma membrane can be measured using NanoDrop method. Each 1 ml elution sample results in mean tyrosine kinase concentration of 233.2 $\mu\text{g/ml}$. Levels of tyrosine kinase isolates from Merino sheep spermatozoa plasma membrane obtained can be used in determining tyrosine kinase supplementation dose in frozen semen diluent medium .

Tyrosine kinases found in human spermatozoa with a molecular weight of 95 kDa has a function for tyrosine phosphorylation and correlated with the occurrence of sperm capacitation and hyperactivation¹⁵. Spermatozoa capacitation allows the spermatozoa membrane binding of to the egg zona pellucida which will further stimulate the sperm to undergo acrosome reaction¹⁶.

Tyrosine kinase isolates whose concentration has been identified can be added to frozen semen diluents media which is expected to trigger an increase in tyrosine kinase receptors on spermatozoa plasma membrane, which further activate transduction signal through increased adenylyl cyclase. Adenylate cyclase may activate CAMP and induces the activity of protein kinase A (PK A). Increased PK will activate tyrosine kinases and therefore tyrosine phosphorylation occurs¹⁷.

Conclusions

Tyrosine kinases can be identified from the plasma membrane of Merino sheep's spermatozoa using SDS-PAGE with a molecular weight of 95.55 kDa. Tyrosine kinase isolation of Merino sheep's spermatozoa plasma membrane can be done by electroelution techniques which produce average concentration of tyrosine kinase isolates of 233.2 $\mu\text{g/ml}$.

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