THE EXTRACTION AND PURIFICATION OF BOAR SPERM SURFACE PROTEIN[†]

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Abstract

Immunological sperm sexing is one of the desirable choices to separate X and Y sperm. The basic concept of immunological technique is based on the different proteins on the surface of X and Y sperms. To investigate the sperm surface proteins, proteomic investigation using 2 dimensional gel (2D-gel) electrophoresis technique has been applied. The initial step, protein extraction is very important. Thus, the suitable strategies for sperm surface protein extraction from unsorted boar spermatozoa surface were considered for further use in 2D-gel analyses. The sperm surface proteins were extracted and then purified using Con-A Sepharose beads. After 22 h incubation, 2.625 mg/ml and 0.186 mg/ml of the total protein can be obtained using extraction solution with and without 0.5% Triton X-100, respectively. The small-sized proteins (~13-16 kDa) were the major products which can be extracted immediately in extraction solution containing Triton X-100. Almost all of the proteins, especially the small-sized products (~13 and 16 kDa), were tightly bound to the Con-A Sepharose beads even in the presence of 800 mM D-glucopyranoside. These results indicated that the major extracted proteins in Triton X-100 solution were glycosylated proteins. In the future, surface proteins from sex sorted sperms (X sperm or Y sperm only) will be extracted by this strategy and then the protein pattern on 2D-gels will be compared.

Keywords: Immunological sexing, spermatozoa, sperm surface protein, sex sorted sperm

Introduction

Sex predetermination of offsprings of agriculturally important animal species could have a highly significant impact on the efforts of producers to reduce production costs (Abeydeera *et al.*, 1998). In a pig, combination of sexed-sorting and cryopreservation of sperms prior to artificial insemination (AI) or *in vitro* fertilization (IVF) followed by embryo transfer have been applied (Bathgate *et al.*, 2007). However, commercialization of sex-sorted sperms using flow cytometry technique still has problems in terms of high economic cost and sperm damage (Seidel G. E., 2003). Therefore, the cheaper and less invasive immunological approaches are highly desirable for the sperm when compared with the flow cytometry

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technique. The basic concept of immunological technique is based on the different proteins on the surface of X and Y sperms (Seidel and Johnson, 1999). Specific proteins on X or Y sperm surface can be a good biomarker for monoclonal antibody production, which is specific to only X or Y sperm. Thus, the investigation of proteins properties from X and Y sperm surface is required.

According to boar sperm surface properties, it has been demonstrated that the ejaculated spermatozoa surface is coated with a number of seminal plasma proteins. These surface proteins have various biochemical activities such as haemagglutination, heparin-binding or zona pellucida-binding (Ohsako *et al.*, 1997). The majority of these proteins are 12–16 kDa glycoproteins that bind to the sperm surface (Caballero *et al.*, 2008).

To investigate the sperm surface proteins, proteomics is an approach used for comparisons of the proteomes which are involved in biological process from difference sources (Strzeżek *et al.*, 2005). The two-dimensional gel (2D-gel) electrophoresis has been applied to separate the protein from each sample for proteomic investigation (Hamady *et al.*, 2005). In the initial step, the protein extraction is a very importance part for this technique. Thus, the suitable strategies for sperm surface protein extraction should be considered.

The objective of this research was to extract proteins from unsorted boar spermatozoa surface for further analyze by 2D-gel. The group of 12–16 kDa glycoproteins that bind to the sperm surface was used as a marker to confirm that the proteins of sperm surface can be extracted. The extracted sperm surface proteins were purified by Con-A Sepharose beads (Concanavalin A coupled to Sepharose; Amersham Biosciences AB, Uppsala, Sweden) to prove that proteins derived by this investigation were glycosylated surface proteins. The extraction method used in this research was able to use to extract boar sperm surface proteins.

Materials and Methods

Sperm Preparation

To separate sperm from seminal plasma, 150 ml of fresh boar sperms were centrifuged at 4,000 rpm, 25°C for 10 min. Then, the sperm pellet was resuspended in 40 ml phosphate buffer saline (PBS) pH 7.4 followed by centrifugation at 4,000 rpm, 25°C for 10 min to wash the sperm pellet. The sperm pellet was washed 8 times and then resuspended in extraction solution to extract sperm surface protein in the next step.

Sperm Surface Protein Extraction

The sperm suspension was directly incubated in a 15-ml centrifuge tube containing 5 ml extraction solution (20 mM Tris-HCl, 1 mM PMSF (phenylmethanesulphonylfluoride), 1X protease cocktail inhibitor (Sigma-Aldrich, Madrid, Spain), 0.5% Triton X-100 pH 7.4, 500 mM NaCl) by shaking on ice for 22 h. For the control experiment, the extraction solution without 0.5% Triton X-100 and 500 mM NaCl was applied.

After 22 h of incubation, the number of sperm cell was determined by heamacytometer. Proteins in the supernatant of each extraction solution, total sperm cell suspension and sperm cell pellet were monitored by 15% SDS-PAGE. Total protein concentration of each sample was analyzed according to Bradford (Bradford, 1976).

Purification of Sperm Surface Protein using Con-A Sepharose Bead

Two hundred ul of the glycan binding Con-A Sepharose 4B beads (Amersham Biosciences AB, Uppsala, Sweden) were equilibrated with 5 ml of 20 mM Tris-HCl, pH 7.4, containing 500 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. Then, 1 ml of each sperm surface protein sample containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$ was applied to the prepared adsorbent and incubated by shaking on ice for 1 h. The adsorbent was allowed to settle and supernatant was collected. The binding steps were repeated 3 times. The residual unbound protein on an adsorbent was eliminated by

washing with 5 ml of washing solution (20 mM Tris-HCl, 500 mM NaCl, 100 mM D-glucopyranoside, pH 7.4). In the step of elution, Con-A Sepharose beads adsorbed with glycosylated protein was incubated on ice with 0.1-1 ml elution buffer (20 mM Tris-HCl, 500 mM NaCl, 800 mM D-glucopyranoside, pH 7.4) for 3 min. The elution steps were repeated to achieve total elution fraction of 2 ml. The fractions of supernatant from binding steps, washing and elution were collected and subjected to 15% SDS-PAGE for protein band detection.

Results and Discussion

Sperm Surface Protein Extraction

After 22 h incubation of unsorted boar sperm (150 ml of approximately 2 x 10^9 cell/ml) in extraction solution with and without (control) 0.5% Triton X-100, 2.625 mg/ml and 0.186 mg/ml of the total protein were obtained, respectively. The results of protein extraction (Figure 1) showed that the high intensity of small-sized protein bands can be detected in extraction solution containing Triton X-100 but

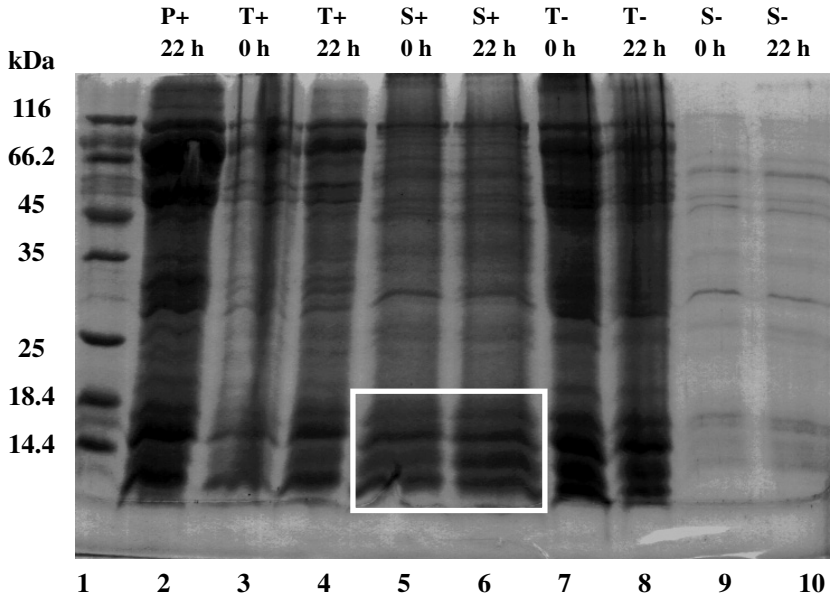

Figure 1. Boar sperm surface protein analysis using 15% SDS-PAGE. Sperm pellet was incubated in extraction solution with (+) or without (-) 0.5% Triton X-100 for 0 and 22 h. P: Sperm pellet; T: Total sperm cell suspension; S: Sperm surface protein in supernatant; lane 1: Protein molecular marker; lane 2: P from 0.5% Triton X-100 + 500 mM NaCl at 22 h; lane 3: T from 0.5% Triton X-100 + 500 mM NaCl at 0 h; lane 4: T from 0.5% Triton X-100 + 500 mM NaCl at 22 h; lane 5: S from 0.5% Triton X-100 + 500 mM NaCl at 0 h; lane 6: S from 0.5% Triton X-100 + 500 mM NaCl at 22 h; lane 7: T from Tris-HCl at 0 h; lane 8: T from Tris-HCl at 22 h; lane 9: S from Tris-HCl at 0 h ; lane 10: S from Tris-HCl at 22 h . The major products of small-sized sperm surface proteins were shown in the box

not in the control without Triton X-100. Similar results have been reported by Ohsako *et al.* (1997). They extracted miniature swine sperm surface proteins using a hypertonic saline solution. They reported that 13 and 16 kDa proteins in the extraction fraction were localized to whole sperm surface (detected on 15% SDS-PAGE). The 13 kDa protein has haemagglutination activity while the 16 kDa does not.

In a boar, the bulk of seminal plasma protein (>90%) belong to the spermadhesin family. They are a group of 12–16 kDa glycoprotein that bind to the sperm surface (Caballero *et al.*, 2008). Spermadhesins, such as AQN (12 kDa), AWN (15 kDa), PSP (14-16 kDa), and DQH (13 kDa) sperm surface proteins, are the most abundant seminal proteins (Calvete *et al.*, 1995; Maňásková and Jonáková, 2008). Heparin binding proteins, AQN-1 and DQH, play a role to stabilize the plasma membrane over the acrosomal vesicle and probably participate in formation of sperm oviductal reservoir (Ekhlasi-Hundrieser *et al.*, 2005; MaHňásková *et al.*, 2007). Non-heparin binding proteins, such as the heterodimer complex of PSP-I/ PSP-II, are mainly localized to the acrosomal area to preserve sperm viability, motility, and mitochondrial activity (Caballero *et al.*, 2006). The results from this experiment confirmed that the small protein band (13-16 kDa) was the seminal plasma proteins which are bound to the sperm surface.

The intensity of protein bands showed that the extracted protein pattern derived from solution containing 0.5% Triton X-100 at 0 and 22 h were not different (Figure 1, lane 5 and 6). Thus, these surface proteins can be extracted immediately after incubated in extraction solution containing Triton X-100.

Purification of Sperm Surface Protein using Con-A Sepharose Beads

Con-A Sepharose beads were applied to the purification of surface protein. Con A Sepharose beads bind molecules containing α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. This affinity adsorbent can be applied to isolate many glycoproteins especially cell surface glycoproteins from detergent-solubilized membranes (Amersham Bioscience, Instruction Note).

To confirm that the sperm surface protein solubilized in the extraction solution containing 0.5% Triton X-100 was glycoprotein, sperm surface proteins in each extraction solution were applied to Con-A Sepharose beads. The protein was then eluted with elution solution containing 800 mM D-glucopyranoside. The purification results of the extracted proteins using Triton X-100 (Figure 2) showed that the small-sized protein (~ 13 and 16 kDa) tightly bound to the Con-A Sepharose bead even in the present of 800 mM D-glucopyranoside. These indicated that the small-sized products were the glycosylated protein.

The patterns of protein band of the samples which eluted from Con-A Sepharose beads were investigated. The results from feedstock extracted with Triton X-100 (Figure 2) and controlled without Triton X-100 (Figure 3) were different. Figure 3 showed that some proteins can be solubilized in the control but at a very low concentration when compared to proteins concentration extracted with Triton X-100. These results suggested that the extracted proteins derived from Triton X-100 were the sperm surface proteins.

However, the purification results showed that the bound proteins could not be eluted from Con-A Sepharose beads even in a high concentration of D-glucopyranoside (Figure 2, lanes 8 and 15). It is possible that these glycosylated proteins are tightly bound to Con-A Sepharose beads and very difficult to elute from the adsorbent. The observation of protein band patterns from purification results (Figure 2) showed that almost all of the proteins solubilized in Triton X-100 solution were able to bind to Con-A Sepharose beads. These results demonstrated that the major proteins present in Triton X-100 solution were the glycosylated surface proteins.

Conclusions

Pre-selected sex of offspring in the livestock reproduction is important for improving reproductive management and reducing reproduction time and cost. Sperm sexing by

immunological method is one of the desirable choices. The advantages of this technique are low processing cost, less invasive for the sperm, and no limitation in the yield of sperms. To separate the proteins from sperm sample for proteomic investigation, 2D-gel has been applied. The suitable strategies of surface protein extraction from boar sperm were investigated. The sperm surface proteins can be extracted by Triton X-100. The small-sized proteins (~13-16 kDa) were the major products which were present in the extraction solution containing 0.5% Triton X-100. These products should be the seminal plasma proteins that bind to the sperm surface. These proteins can be extracted immediately after being incubated in extraction solution containing Triton X-100. The purification of these sperm surface protein using Con-A Sepharose beads indicated that most proteins solubilized in Triton X-100 solution were the glycosylated proteins. Thus, mostly they are able to bind to Con-A Sepharose beads. Therefore, this research was able to extract sperm surface proteins.

For further work, surface proteins from sex sorted sperms (X sperm or Y sperm only) will be extracted and then the protein patterns on 2D-gel will be compared. The different proteins from X and Y sperms will be identified as protein markers. The long term goal is to produce monoclonal antibody to the X- and Y- specific sperm surface proteins for sperm sexing application by immunological technique.

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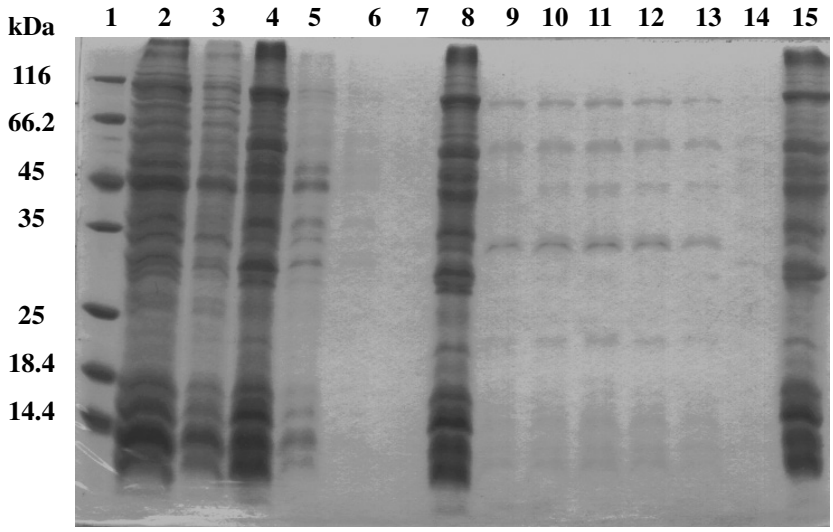

Figure 2. The SDS-PAGE (15%) analysis of boar sperm surface protein purification fractions using Con-A Sepharose beads. Boar sperm surface protein was extracted by extraction solution containing 0.5% Triton X-100 and 500 mM NaCl for 22 h. lane 1: Protein molecular marker; lane 2: soluble fraction (s); lane 3: supernatant fraction from binding step; lane 4: Con-A Sepharose beads after binding step; lanes 5-7: washing fraction numbers 2, 4, and 6; lane 8: Con-A Sepharose beads after washing step; lanes 9-14: elution fraction numbers 1, 2, 3, 4, 6, 8; lane 15: Con-A Sepharose beads after elution step

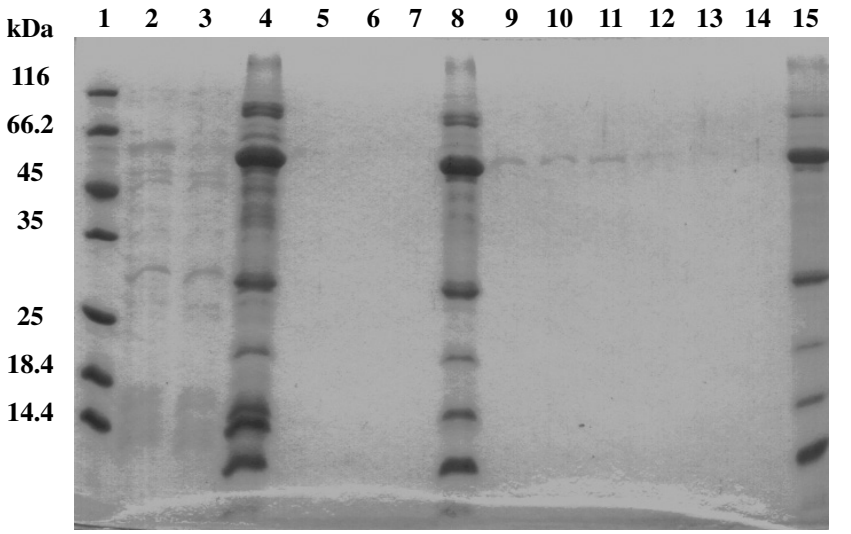

Figure 3. The SDS-PAGE (15%) analysis of boar sperm surface protein purification fractions using Con-A Sepharose beads. Boar sperm surface protein was extracted by extraction solution without 0.5% Triton X-100 for 22 h (negative control). lane 1: Protein molecular marker, lane 2: soluble fraction (s); lane 3: supernatant fraction from binding step; lane 4: Con-A Sepharose beads after binding step; lanes 5-7: washing fraction numbers 2, 4, and 6; lane 8: Con-A Sepharose beads after washing step; lanes 9-14: elution fraction numbers 1, 2, 3, 4, 6, 8; lane 15: Con-A Sepharose beads after elution step

References

Abeydeera, L. R., Johnson, L. A., Welch, G. R., Wang, W. H., Boquest, A. C., Cantley, T. C., Rieke, A., and Day, B. N. (1998). Birth of piglets preselected for gender following *in vitro* fertilization of *in vitro* matured pig oocytes by X and Y chromosome bearing spermatozoa sorted by high speed flow cytometry. Theriogenology, 50: 981-988.

Bathgate, R., Morton, K. M., Eriksson, B. M., Rath, D., Seig, B., Maxwell, W. M. C., and Evans, G. (2007). Non-surgical deep intra-uterine transfer of *in vitro* produced porcine embryos derived from sex-sorted frozen–thawed boar sperm. Anim. Reprod. Sci., 99: 82–92.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Caballero, I., Vázqez, J. M., Garciá, E. M., Roca, J., Martínez, E. A., Calvete, J. J., Sanz, L., Ekwall, H., and Rodríguez-Martínez, H. (2006). Immunolocalization and possible functional role of PSP-I/PSP-II heterodimer in highly extended boar spermatozoa. J. Andrology, 27(6): 766-773.

Caballero, I., Vazquez, J. M., Garc1á, E. M., Parrilla, I., Roca, J., Calvete, J. J., Sanz, L., and Martínez, E. A. (2008). Major proteins of boar seminal plasma as a tool for biotechnological preservation of spermatozoa. Theriogenology, 70(8): 1352-1355.

Calvete, J. J., Mann, K., Schäifer, W., Raida, M., Sanz, L., and Töpfer-Petersen, E. (1995). Boar spermadhesin PSP-II: location of posttranslational modifications, heterodimer formation with PSP-I

glycoforms and effect of dimerization on the ligand-binding capabilities of the subunits. FEBS Lett., 365: 179-182.

Ekhlasi-Hundrieser, M., Gohr, K., Wagner, A., Tsolova, M., Petrunkina, A., and Töpfer-Petersen, E. (2005). Spermadhesin AQN1 is a candidate receptor molecule involved in the formation of the oviductal sperm reservoir in the pig. Biol. Reprod., 73: 536-545.

Hamady, M., Cheung, T. H. T., Resing, K., Cios, K. J., and Knight, R. (2005). Key challenges in proteomics and proteoinformatics. Eng. Med. Biol. Mag., 24(3): 34-40.

Maňásková, P., Pěknicová, J., Elzeinová, F., Tichá, M., and Jonáková, V. (2007). Origin, localization binding abilities of boar DQH sperm surface protein tested by specific monoclonal antibodies. J. Reprod. Immunol., 74: 103-113.

Maňásková, P., and Jonáková, V. (2008). Localization of porcine seminal plasma (PSP) proteins in the boar reproductive tract and spermatozoa. J. Reprod. Immunol., 78: 40-48.

Ohsako, S., Ikoma, E., Nakanishi, Y., Nagano, R., Matsumoto, M., and Nishinakagawa, H. (1997). Isolation of a miniature swine seminal plasma haemagglutinin from the sperm surface. J. Reprod. Dev., 43(4): 311-319.

Seidel, G. E., and Johnson, L. A. (1999). Sexing mammalian sperm-overview. Theriogenology, 52: 1267-1272.

Seidel, G. E. (2003). Sexing mammalian spermintertwining of commerce, technology, and biology. Anim. Reprod. Sci., 79(3-4): 145-156.

Strzeżek, J., Wysocki, P., Kordan, W., Kukliňska, M., Mogielnicka, M., Soliwoda, D., and Fraser, L. (2005). Proteomics of boar seminal plasma-current studies and possibility of their application in biotechnology of animal reproduction. Reprod. Biol., 5(3): 279-290.