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# In Vitro Developmental Competence of In Vitro-Matured Bovine Oocytes Fertilized and Cultured in Completely Defined Media[1]

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## ABSTRACT

**The objective was to establish an in vitro system in which bovine oocytes can be matured, fertilized, and cultured up to the blastocyst stage without support of serum, BSA, or somatic cells. Media consisted of modified tissue culture medium 199 (mTCM 199) with ovine LH (oLH) for maturation (IVM), experimental alterations of modified defined medium (mDM) for sperm selection and insemination (IVF), and citrate + synthetic oviductal fluid + nonessential amino acids (c-SOF+NEA) for zygote/embryo culture (IVC). Effects of heparin, BSA, polyvinyl alcohol (PVA), penicillamine (P), Hepes, and sodium bicarbonate ($NaHCO_3$) were studied. Results included proportions of oocytes that cleaved by 48 h and that reached morulae by 120 h, blastocysts by 168 h, and expanded blastocysts by 216 h postinsemination (pi). Best results were obtained when the IVF medium included 0.5 mg P + 1.0 mg PVA per milliliter with no more than 10 mM Hepes, and when 3.0 mg PVA/ml and 10 mM Hepes were present for IVC. Different concentrations of $NaHCO_3$, up to 50 mM from 25 mM, during IVF did not alter results. Embryos produced in defined conditions yielding the best results remained viable after vitrification as evidenced by continued development in vitro for 96 h postthawing. Bovine oocytes matured in defined medium supplemented with LH were fertilized and cultured up to the blastocyst stage in chemically defined conditions that afforded results comparable to those reported earlier after inclusion of serum, BSA, and/or somatic cells.**

## INTRODUCTION

The development of defined bovine embryo culture systems holds promise for ongoing efforts in reproductive physiology and for utility in the embryo transfer industry. Although a variety of embryo culture systems are employed for in vitro embryo production, systems that include oviduct epithelial cells [1–3], granulosa cells [4–6], or trophoblastic vesicles [2, 7] are lacking adequate definition to assure quality control and repeatability. To eliminate excessive variability, and to lead to better understanding of preimplantational development, simplified culture systems have been employed [8–14]. Recent reports on defined in vitro conditions have assisted the understanding of molecular mechanisms capable of promoting or impeding early embryonic development [15–17]. Amino acids [8, 10, 13, 18–20], energy substrates [21, 22], citrate [13, 23], and vitamins [10, 22, 24] can profoundly influence embryonic development in vitro.

Serum and BSA are among the most common components of media for mammalian embryo culture. Serum, which contains hormones, growth factors, vitamins, chelators for heavy metal ions, peptides, proteins, and an array of defined and nondefined molecules [25], is generally included as the fixed nitrogen source for the preimplantation embryo [17]. However, serum was found to have a biphasic influence on development of bovine embryos, being deleterious to the first cleavage division but stimulatory for blastocyst development [11, 26]. In early mouse embryo experiments, BSA ameliorated water quality when embryotoxicity was a problem [27]. BSA may be contaminated with several extraneous peptides, energy substrates, and growth factors [28, 29]. Different batches of commercially available BSA might inhibit or stimulate embryonic development [23, 26]. BSA has been commonly replaced by synthetic macromolecules, polyvinyl alcohol (PVA) or polyvinylpyrrolidone (PVP), when chemically defined conditions are desired [13, 30, 31].

Among other components investigated during in vitro maturation, fertilization, and culture (IVMFC) are sodium bicarbonate ($NaHCO_3$) [32, 33]; insulin, transferrin, and selenium [34]; growth factors [35, 36]; gas atmosphere [37]; glucose [38]; penicillamine (P) [39]; antioxidants [40]; and water quality [41]. However, completely defined conditions have been unavailable for definitive testing; either serum or BSA has been included for one or more of the steps composing the IVMFC procedure. All incompletely defined biological additives including heparin treatment for sperm capacitation should be eliminated for a more accurate assessment of influences of experimental treatments on IVMFC outcome.

The objectives of this study were to define conditions for sperm treatment, in vitro fertilization (IVF), and in vitro culture (IVC) without compromising the quantity and quality of blastocyst production from immature oocytes via the entire IVMFC system. Influences of heparin, BSA, PVA, P, $NaHCO_3$, and Hepes during IVF and IVC were studied, and products of IVMFC were challenged by vitrification to assess competency for continuing in vitro development.

## MATERIALS AND METHODS

### *Oocyte Recovery and In Vitro Maturation (IVM)*

Oocyte collection and IVM were carried out as reported earlier [13]. Briefly, bovine ovaries were obtained within 15–30 min after slaughter. Follicles 2–5 mm in diameter were aspirated with a 10-cc syringe fitted with an 18-gauge needle. Follicular aspirates were collected into a 50-ml plastic flask (Falcon 3014; Becton Dickinson Labware, Franklin Lakes, NJ) and then held at 30–33°C during transport to the laboratory (~2.5 h). Oocyte selection [42] and all other manipulations were carried out at 39°C. Oocytes with 3–4 layers of cumulus cells and having homogenous cytoplasm were washed through PBS with 0.4 mg/ml polyvinyl alcohol (P-8136; Sigma Chemical Co., St. Louis, MO) and transferred into 100-μl drops of mTCM 199 (M-3769, Sigma) with additions for IVM during a 24-h

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31. Horvath TL, Naftolin F, Leranth C. β-Endorphin innervation of dopamine neurons in the rat hypothalamus: a light and electron microscopic double immunostaining study. Endocrinology 1992; 131:1547–1555.
32. Conover CD, Kuljis RO, Rabii J, Advis JP. Beta-endorphin regulation of luteinizing hormone-releasing hormone release at the median eminence in ewes: immunocytochemical and physiological evidence. Neuroendocrinology 1993; 57:1182–1195.
33. Lehman MN, Karsch FJ. Do gonadotropin-releasing hormone, tyrosine hydroxylase, and β-endorphin-immunoreactive neurons contain estrogen receptors? A double-label immunocytochemical study in the Suffolk ewe. Endocrinology 1993; 133:887–895.
34. Kineman RD, Leshin LS, Crim JW, Rampacek GB, Kraeling RR. Localization of luteinizing hormone-releasing hormone in the forebrain of the pig. Biol Reprod 1988; 39:665–672.
35. Kineman RD, Kraeling RR, Crim JW, Leshin LS, Barb CR, Rampacek GB. Localization of proopiomelanocortin (POMC) immunoreactive neurons in the forebrain of the pig. Biol Reprod 1989; 40:1119–1126.
36. Leshin LS, Kineman RD, Barb CR, Kiser TE, Rampacek GB, Kraeling RR. Tyrosine hydroxylase (TH) immunoreactive neurons in the hypothalamus of cattle and pigs. Soc Neurosci 1989; 15:587 (abstract).
37. Cacicedo L, Sanchez-Franco F. Direct action of opioid peptides and naloxone on gonadotropin secretion by cultured rat anterior pituitary cells. Life Sci 1986; 38:617–625.
38. Blank MS, Fabbri A, Catt KJ, Dufau ML. Inhibition of luteinizing hormone release by morphine and endogenous opiates in cultured pituitary cells. Endocrinology 1986; 118:2097–2101.
39. Chao CC, Moss GE, Malven PV. Direct opioid regulation of pituitary release of bovine luteinizing hormone. Life Sci 1986; 39:527–534.
40. Barb CR, Barrett JB, Wright JT, Kraeling RR, Rampacek GB. Opioid modulation of LH secretion by pig pituitary cells *in vitro*. J Reprod Fertil 1990; 90:213–219.
41. Miki N, Sonntag WE, Forman LJ, Meites J. Suppression by naloxone of rise in plasma growth hormone and prolactin induced by suckling. Proc Soc Exp Biol Med 1981; 168:330–333.
42. Knight PG, Howles CM, Cunningham FJ. Evidence that opioid peptides and dopamine participate in the suckling-induced release of prolactin in the ewe. Neuroendocrinology 1986; 44:29–35.
43. Baumann MH, Rabii J. Inhibition of suckling-induced prolactin release by μ- and k-opioid antagonists. Brain Res 1991; 567:224–230.
44. Johnson DW, Barnes MA, Akers RM, Pearsson RE. Exogenous opioids increase plasma prolactin in holstein calves primarily via a dopaminergic mechanism. J Anim Sci 1991; 69:4545–4551.
45. Soaje M, Deis RP. A modulatory role of endogenous opioids on prolactin secretion at the end of pregnancy in the rat. J Endocrinol 1994; 140:97–102.
46. Smith MS. The relative contribution of suckling and prolactin to the inhibition of gonadotropin secretion during lactation in the rat. Biol Reprod 1978; 19:77–83.
47. Sirinathsinghji DJS, Martini L. Effects of bromocriptine and naloxone on plasma levels of prolactin, LH and FSH during suckling in the female rat: responses to gonadotrophin releasing hormone. J Endocrinol 1984; 100:175–182.
48. Kremer JAM, Borm G, Schellekens LA, Thomas CMG, Rolland R. Pulsatile secretion of luteinizing hormone and prolactin in lactating and nonlactating women and the response to naltrexone. J Clin Endocrinol & Metab 1991; 72:294–300.
49. Bevers MM, Willemse AH, Kruip TAM. The effect of bromocriptine on luteinizing hormone levels in the lactating sow: evidence for a suppressive action by prolactin and the suckling stimulus. Acta Endocrinol 1983; 104:261–265.
50. De Rensis F, Hunter M, Foxcroft GR. Suckling-induced inhibition of LH secretion and follicular development in the early postpartum sow. Biol Reprod 1993; 48:964–969.
51. De Passillé AMB, Rushen J, Foxcroft GR, Aherne FX, Schaefer A. Performance of young pigs: relationships with periparturient progesterone, prolactin, and insulin of sows. J Anim Sci 1993; 71:179–184.
52. Pharazyn A, Foxcroft GR, Aherne FX. Temporal relationship between plasma progesterone concentrations in the utero-ovarian and jugular veins during early pregnancy in the pig. Anim Reprod Sci 1991; 26: 323–332.
53. Shaw HJ, Foxcroft GR. Relationships between LH, FSH and prolactin and reproductive activity in the weaned sow. J Reprod Fertil 1985; 75:17–28.
54. Cosgrove JR, Booth PJ, Foxcroft GR. Opioidergic control of gonadotrophin secretion in the prepubertal gilt during restricted feeding and realimentation. J Reprod Fertil 1991; 91:277–284.
55. SAS. SAS User's Guide. Statistics, Cary, NC: Statistical Analysis System Institute Inc.; 1988.
56. McMillen IC, Deayton JM. Effect of morphine and naloxone on plasma prolactin concentrations in the fetal sheep and pregnant ewe during late gestation. Neuroendocrinology 1989; 49:286–290.
57. Rund LA, Thompson FN, Byerley DJ, Kiser TE. Failure of naloxone to stimulate luteinizing hormone secretion during pregnancy and steroid treatment of ovariectomized beef cows. Biol Reprod 1990; 42: 619–624.
58. Kalra SP, Allen LG, Sahu A, Kalra PS, Crowley WR. Gonadal steroids and neuropeptide Y-opioid-LHRH axis: interactions and diversities. J Steroid Biochem 1988; 30:185–193.
59. Haynes NB, Lamming GE, Yang K-P, Brooks AN, Finnie AD. Endogenous opioid peptides and farm animal reproduction. In: Milligan SR (ed.), Oxford Reviews of Reproductive Biology 1989; 11:111–145.
60. Barb CR, Kraeling RR, Rampacek GB, Whisnant CS. Opioid inhibition stimulates luteinizing hormone and prolactin secretion in gilts. Domest Anim Endocrinol 1985; 2:93–98.
61. Barb CR, Rampacek GB, Kraeling RR, Estienne MJ, Taras E, Estienne CE, Whisnant CS. Absence of brain opioid peptide modulation of luteinizing hormone secretion in the prepubertal gilt. Biol Reprod 1988; 39:603–609.
62. Smith CA, Almond GW. Effects of season and stage of gestation on luteinizing hormone release in gilts. Can J Vet Res 1991; 55:294–297.
63. Szafranska B, Ziecik AJ. Effect of bromocriptine administration during mid-pregnancy on the ovary function in the pig. Exp Clin Endocrinol 1990; 96:317–320.
64. Tennekoon KH, Lenton EA. Synchronous secretion of LH and prolactin during the normal menstrual cycle. Asia-Oceania J Obstet Gynaecol 1993; 19:101–107.
65. Lafuente A, Marcó J, Esquifino AI. Pulsatile prolactin secretory patterns throughout the oestrous cycle in the rat. J Endocrinol 1993; 137: 43–47.
66. Edwards HE, Reburn CJ, Wynne-Edwards KE. Daily patterns of pituitary prolactin secretion and their role in regulating maternal progesterone concentrations across pregnancy in Djungarian hamster (*Phodopus campbelli*). Biol Reprod 1995; 52:814–823.
67. Foxcroft GR, Van De Wiel DFM. Endocrine control of the oestrous cycle. In: Cole DJA, Foxcroft GR (eds.), Control of Pig Reproduction 1982; 8:161–177.
68. Wiesak T. Influence of hCG, LH and PRL on progesterone, oestradiol, and testosterone secretion by luteal cells from pigs during pregnancy and pseudopregnancy. Olsztyn, Poland: University of Agriculture and Technology; 1985. Ph.D. Thesis.
69. Szafranska B, Grazul-Bilska AT, Przala J. Effect of LH and prolactin on steroid secretion by perifused luteal tissue from pregnant gilts with immunoneutralization of LH. Arch Vet Pol 1992; 32:7–15.
70. Rolland R, Gunsalus GL, Hammond JM. Demonstration of specific binding of prolactin by porcine corpora lutea. Endocrinology 1976; 98:1083–1091.
71. Jammes H, Schirar A, Djiane J. Differential patterns in luteal prolactin and LH receptors during pregnancy in sows and ewes. J Reprod Fertil 1985; 73:27–35.
72. Cook B, Kaltenbach CC, Norton HW, Nalbandov AV. Synthesis of progesterone *in vitro* by porcine corpora lutea. Endocrinology 1967; 81:573–584.
73. Gregoraszczuk EL. Different response of porcine large and small luteal cells to PRL in terms of progesterone and estradiol secretion in vitro. Exp Clin Endocrinol 1990; 96:234–237.
74. Taverne M, Bevers M, Bradshaw JMC, Dieleman SJ, Willemse AH, Porter DG. Plasma concentrations of prolactin, progesterone, relaxin and oestradiol-17β in sows treated with progesterone, bromocriptine or indomethacin during late pregnancy. J Reprod Fertil 1982; 65:85–96.

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# Electrophoretic Characterization of Boar Epididymal Antiagglutinin[1]

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## ABSTRACT

**Boar epididymal antiagglutinin, previously shown to inhibit sperm head-to-head agglutination, was purified from cauda epididymal plasma by precipitation with ammonium sulfate, anion-exchange chromatography, and reverse-phase HPLC, and was characterized by electrophoretic and membrane blotting techniques. Blotting techniques, using the ECL Glycoprotein Detection System (Amersham Life Science, Buckinghamshire, UK) and wheat germ agglutinin (WGA)-peroxidase, established the presence of sialic acid residues on purified antiagglutinin. Removal of sialic acid residues from antiagglutinin greatly reduced its immunoreactivity with the specific antiserum. Further purification by two-dimensional PAGE established the presence of one major and two minor forms that cross-reacted with the antiserum, with only the major form reacting with WGA-peroxidase. Extracts of washed epididymal spermatozoa contained a polypeptide with the same electrophoretic mobility as the major form. Additionally, the antiserum detected cross-reacting material in seminal plasma and in extracts from ejaculated spermatozoa. When spermatozoa were incubated under conditions shown to promote capacitation, the cross-reacting material could not be detected in sperm extracts. These results are consistent with the following conclusions: 1) antiagglutinin contains sialic acid residues that may be related to its immunoreactivity and molecular heterogeneity, and 2) either sperm-bound antiagglutinin is released or its epitope recognized by the antiserum is altered after ejaculation and in vitro capacitation.**

## INTRODUCTION

Boar spermatozoa acquire the capacity for progressive motility [1, 2], to bind tightly to zona pellucida [3] and to the egg plasma membrane [1, 4] during epididymal transit. Most cells acquire fertilizing ability in the distal corpus and proximal cauda epididymides [5, 6]. In the cauda epididymidis, sperm fertilizing ability appears to be temporally reduced by the addition of stabilizing factors for effective storage of spermatozoa. These factors have been identified in boars [7, 8], but not characterized to the extent to which the acrosome stabilizing factor of rabbits has been [9, 10]. The epididymal environment, which provides a favorable environment for sperm to acquire fertilizing ability and for sperm storage by epithelial secretion and absorption [11–13], is established by the interaction between epididymal proteins and spermatozoa [14]. Russell et al. [15] and Dacheux et al. [16] compared SDS-PAGE and two-dimensional PAGE patterns of fluids collected from different regions of the boar epididymis. However, only limited data are available on the detailed characterization of the specific boar epididymal proteins.

Bostwick et al. [17] identified a 133-kDa protein in cauda epididymal fluid whose distribution on spermatozoa changed during epididymal transit. Dacheux and Dacheux [18–20] observed the immunohistochemical distribution of a 250-kDa antiagglutinin and its androgen-dependent secretion. Okamura et al. [21, 22] showed the distribution of a 135-kDa epididymal fluid protein (mannosidase) and characterized its interaction with spermatozoa. We previously reported that a 25-kDa epididymal protein (antiagglutinin) effectively reduced head-to-head agglutination of spermatozoa and maintained sperm progressive motility in vitro [23]. The aims of this study were to further characterize the 25-kDa epididymal antiagglutinin by refined electrophoretic techniques, to test for the presence and importance of sialic acid residues on the molecule, and to assess the effect of ejaculation and in vitro capacitation on its retention on the spermatozoal surface.

## MATERIALS AND METHODS

### *Sample Preparation*

Boar epididymal fluid was recovered from 11 cauda epididymides by retrograde displacement with gentle air pressure after a needle attached to a syringe was inserted into the epididymal duct [4]. Most of the fluid was centrifuged at 10 000 × *g* for 15 min at 4°C to obtain epididymal plasma. Epididymal spermatozoa were recovered from the balance of the fluid by a fourfold dilution with PBS (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 12H_2O$, and 1.5 mM $KH_2PO_4$) containing 0.2 mM PMSF (Sigma Chemical Co., St. Louis, MO; PBSp [24]) followed by centrifugation at 400 × *g* for 5 min at room temperature. After removal of the supernatant, epididymal spermatozoa were washed twice (5 min each) in PBSp by centrifugation at 400 × *g* at room temperature. In addition, they were rewashed on a discontinuous gradient of Percoll (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) [25] prepared with PBSp. The resulting sperm pellets were resuspended in ice-cold PBS containing 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml phosphoramidon, 1 mM EDTA disodium salt, 1 mM PMSF, and 1% Nonidet P-40 (NP-40) ($8.0 \times 10^8$ cells/ml; all protease inhibitors and NP-40 were from Sigma) and were then incubated in ice for 2 h. After the incubation, the sperm suspensions were centrifuged at 10 000 × *g* for 15 min at 4°C to obtain the supernatants (sperm extracts).

Sperm-rich fractions from ejaculates obtained from 5 mature boars by the manual method were centrifuged at 400 × *g* for 5 min at room temperature. The supernatants were recentrifuged at 10 000 × *g* for 15 min at 4°C to obtain seminal plasma. The spermatozoa after the first centrifugation were washed twice (5 min each) in PBS containing 0.1% polyvinyl alcohol ($M_r$ 30 000–70 000, Sigma)

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by centrifugation at 400 × $g$. Some of the cells were rewashed on a discontinuous gradient of Percoll and then resuspended in ice-cold PBSp (8.0 × $10^8$ cells/ml). The remaining cells were resuspended (2.0 × $10^8$ cells/ml) in tissue culture medium 199 (TCM 199; Nissui, Tokyo, Japan) containing 100 μg/ml sodium pyruvate, 550 μg/ml glucose, 900 μg/ml calcium lactate, 50 μg/ml streptomycin (Meiji Seika, Tokyo, Japan), 100 μg/ml penicillin (Meiji Seika, Tokyo, Japan), 3.87 mg/ml sodium bicarbonate, and 10% (v/v) fetal calf serum (Gibco BRL, Gaithersburg, MD) (pH 7.8) and incubated in the sealed tube for 4 h at 37°C to induce capacitation [26, 27]. After incubation, spermatozoa were removed from the medium by centrifugation and then resuspended in ice-cold PBSp, as described above.

### *Electrophoresis and Electrotransfer*

For one-dimensional SDS-PAGE, each sample was diluted with an equal volume of double-strength sample buffer (pH 6.8) composed of 125 mM Tris-HCl, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.02% bromophenol blue [28], and then heated for 3 min in a boiling water bath. Samples of ejaculated spermatozoa were clarified after this treatment by centrifugation at 10 000 × $g$ for 15 min at 4°C to remove sperm debris. For two-dimensional PAGE, the samples containing 9 M urea (Pharmacia) were mixed with an equal volume of O'Farrel lysis buffer [29] composed of 9.5 M urea, 2% NP-40, 2% Ampholine (pH 3.5–10) (Pharmacia), and 4% 2-mercaptoethanol. For the Multiphor II Electrophoresis System (Pharmacia), which was used to confirm the exact isoelectric point (pI) of the purified antiagglutinin, the samples containing 9 M urea were mixed with an equal volume of the buffer composed of 9 M urea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS; Sigma), 1% Pharmalyte (pH 3–10) (Pharmacia), 65 mM dithiothreitol (Sigma), and 0.1% bromophenol blue.

One-dimensional SDS-PAGE was performed by use of a 10–15% liner acrylamide gradient gel with Laemmli's buffer system [28]. Two-dimensional PAGE was performed as described by O'Farrel [29] with minor modifications, as follows. Briefly, the samples were applied on the gel rods. Isoelectric focusing (IEF) using final concentrations of 2% Ampholine (pH 3.5–10) and 0.5% Ampholine (pH 4–6) (Pharmacia) were carried out at 300 V for 18 h and then at 450 V for 1 h. The pH profile calibration in IEF gel was done by slicing a blank gel and eluting the 0.5-mm sections in distilled water for 2 h. After IEF, the gels were equilibrated in single-strength sample buffer of SDS-PAGE for 20 min and then loaded on a 10–15% linear acrylamide gel. The second run was carried out at 20 mA for 30 min and then at 40 mA for 3–4 h. For the Multiphor II Electrophoresis System, purified antiagglutinin (2 μg), treated as described above, was loaded on a Drystrip (Pharmacia) that had been rehydrated with a buffer composed of 8 M urea, 5% glycerol, 0.5% CHAPS, 1% Pharmalyte (pH 3–10), and 0.001% orange G. The first run was carried out at 150 V for 45 min and then at 1900 V for 18 h. After equilibration in a buffer (50 mM Tris-HCl [pH 6.8], 6 M urea, 30% glycerol, and 1% SDS) containing 16.2 mM dithiothreitol for 20 min and then in the same buffer containing 143 mM iodoacetamide for 10 min, the strip was loaded on the 12.5% acrylamide gel. The second run was carried out at 20 mA for 2–3 h.

Colorless or prestained SDS-PAGE standards (Bio-Rad Laboratories, Hercules, CA; Promega, Madison, WI; Gibco BRL) were used as molecular weight standards. For the Multiphor II Electrophoresis System, carbonic anhydrase (Pharmacia) was used as a standard of pI (4.8–6.7).

The gels for staining were fixed in 15% ethanol-25% methanol-10% acetic acid fixative for 1–2 h, and then were silver-stained. In the gels for Western blotting, detection of sialic acids, and lectin blotting, proteins were transferred to a nitrocellulose membrane (Hybond-C super; Amersham Life Science, Buckinghamshire, UK) or a polyvinylidene difluoride (PVDF) membrane (Hybond-PVDF, Amersham) in a semi-dry transfer cell for 1 h at 2.0 mA/cm$^2$ in the transfer buffer [30].

### *Preparation of Antiserum against the Antiagglutinin*

The antiagglutinin for immunization was purified from epididymal plasma as described previously [23]. A rabbit was immunized with an injection (s.c.) of 30 μg of purified antiagglutinin emulsified in Freund's complete adjuvant (Wako Pure Chemical Industries LTD, Osaka, Japan), followed by a second injection (s.c.) of 15 μg antigen emulsified in Freund's incomplete adjuvant (Wako) 4 wk later. Blood was collected 1 wk after the second injection, and an antiserum was prepared for the following experiments.

### *Western Blotting*

The blotted membrane was blocked with 5% skim milk (Carnation, Glendale, CA) in PBS containing 0.1% Tween 20 (Sigma) (PBS-Tween) for 1 h. The antiserum against the antiagglutinin was diluted (1:250 000) with PBS-Tween containing 1% skim milk and was incubated with the membrane for 2 h. After being washed three times in PBS-Tween for 10 min each, the membrane was blocked in PBS-Tween containing 5% skim milk for 30 min and then treated with horseradish peroxidase-labeled donkey anti-rabbit immunoglobulins (1:1000; Amersham) in the blocking buffer for 1 h. After the membrane was washed three times, peroxidase activity was visualized by means of the ECL Western Blotting Detection System (Amersham).

### *Lectin Blotting*

Lectin staining was performed as described by Kijimoto-Ochiai et al. [31, 32], with some modifications. In brief, the blotted nitrocellulose membrane was washed twice (10 min each) in Tris-Tween-saline (pH 7.4) composed of 10 mM Tris-HCl, 0.05% Tween 20, and 0.15 M NaCl, and blocked with 1% BSA (Fraction V, Seikagakukogyo, Tokyo, Japan) in Tris-Tween-saline for 1 h. Wheat germ agglutinin (WGA)-peroxidase reagent (Honen Co., Tokyo, Japan) was diluted with Tris-Tween-saline to a final concentration of 0.5 μg protein/ml. The membrane was incubated with the diluted lectin for 1 h at 4°C, and then washed four times (15 min each) in Tris-Tween-saline. The reactive spots were visualized by use of the ECL Western Blotting Detection System.

### *Purification of the Antiagglutinin*

A new method for purification of antiagglutinin is described here. Briefly, the proteins of epididymal plasma (10 ml) were precipitated with 65% ammonium sulfate solution at 4°C and separated from the supernatant by centrifugation at 17 210 × $g$ for 20 min at 4°C. After resuspension in 100 ml of 5 mM Tris-HCl (pH 7.4), the protein solution was dialyzed against 20 mM Tris-HCl (pH 8.0) for 12 h and then against 40 mM Tris-HCl (pH 9.0) for 24 h, concen-

trated by use of an ultrafilter (Cenriflo Membrane Cone Type CF25; Amicon, Beverly, MA), and applied to a DEAE-Sephacel column (2.6 cm × 10 cm; Pharmacia) equilibrated in 40 mM Tris-HCl. Proteins were eluted with a linear gradient obtained with 200 ml 40 mM Tris-HCl and 200 ml 40 mM Tris-HCl containing 1 M NaCl at a flow rate of 14.5 ml/h. Fractions (3.0 ml) were collected, and a portion of each fraction was used for SDS-PAGE and subsequent Western blotting. The fractions containing the antiagglutinin were pooled, concentrated as described above, divided into 10–15 samples (0.5 mg protein each), and stored at −20°C until further purification. Each of the samples was loaded on a reverse-phase HPLC C-18 column (1.0 cm × 25 cm, Vydac 201TP $C_{18}$ Specialty Reverse Phase Column, The Separation Group, Hesperia, CA) equilibrated in 0.1% trifluoroacetic acid (TFA, Sigma) in water. Proteins were eluted at 1.0 ml/min with a gradient of 0.1% TFA in water and acetonitrile (ACN; Fisher Scientific, Fair Lawn, NJ), first isocratically (0% ACN) for 30 min, then at 0–70% ACN for 70 min. Fractions (1.0 ml) were collected, and a portion of each fraction was used for SDS-PAGE and subsequent Western blotting. The fraction containing the antiagglutinin (usually one fraction) was separated from the solvent by freeze-drying and then was loaded on another reverse-phase HPLC C-18 column (0.48 cm × 25 cm, Spherical Dynamax-300A C18; Rainin Instrument Co. Ins., Woburn, MA) and eluted at 0.7 ml/min with a gradient of 0.1% TFA in water and ACN, first isocratically (0% ACN) for 15 min, then at 0–30% ACN for 30 min, and at 30–50% ACN for 40 min. The fractions of the antiagglutinin were concentrated by use of an ultrafilter (Centricon-SR3, Amicon).

*Detection of Sialic Acid Residues in the Antiagglutinin Transferred to the Membrane*

Sialic acid residues of the antiagglutinin were detected by using the ECL Glycoprotein Detection System (Amersham). In brief, the blotted PVDF membrane was incubated in phosphate buffer (pH 7.0) composed of 42.9 mM $Na_2HPO_4$ $7H_2O$, 21.5 mM $NaH_2PO_4$ $H_2O$, and 100 mM NaCl for 10 min at 0°C (in wet ice) and then in ice-cold 100 mM acetate buffer (pH 5.5) containing 1 mM sodium metaperiodate for 20 min at 0°C in the dark. In control experiments, sodium metaperiodate-free acetate buffer was used. After three 10-min washes in the phosphate buffer 0°C, the membrane was incubated in 20 ml acetate buffer containing 4 µl of 5.0 M biotin hydrazide stock solution for 1 h at room temperature. The membrane was washed three times and blocked with 5% blocking agent in the phosphate buffer. Horseradish peroxidase-labeled streptavidin was added to the phosphate buffer (1:1000) and then incubated with the membrane for 30 min. After three washes, peroxidase activity was visualized by the ECL Western Blotting Detection System.

*Chemical Removal of Sialic Acid Residues from Proteins Transferred to the Membrane*

The blotted PVDF membrane was treated in 25 mM $H_2SO_4$ for 1 h at 80°C [31]. After this treatment, the membrane was washed three times (10 min each) in PBS.

*Enzymatic Removal of Sialic Acid Residues from the Proteins*

Epididymal plasma was diluted with 100 mM citrate buffer (pH 5.0) to a protein concentration of 5 mg/ml.

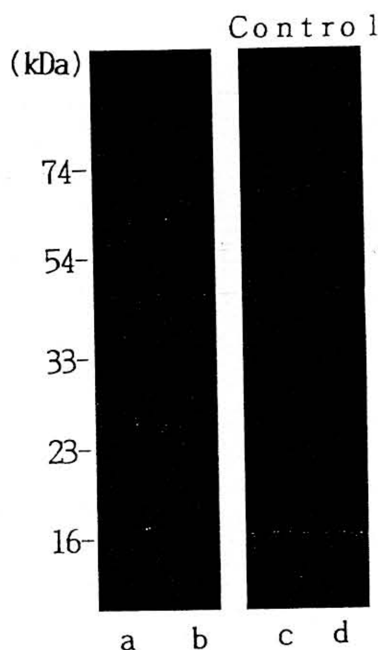



FIG. 1. SDS-PAGE of epididymal plasma (5 µg protein each, lanes a and c) and purified antiagglutinin (1 µg protein each, lanes b and d) immunodetected by antiserum against antiagglutinin (1:250 000; **left**) or preimmune serum (1:10 000; control; **right**).

Neuraminidase (from *Vibrio cholerae*; Boehringer Mannheim Co., Indianapolis, IN) was added to the diluted epididymal plasma and then incubated for 20 h at 37°C. The final concentration of neuraminidase was 0.5 U/ml. In the control experiment, enzyme was added after an incubation in a boiling water bath for 15 min. After the treatment, the samples were frozen at −20°C until used.

*Protein Concentrations*

Protein concentrations were determined according to a dye-binding assay, with BSA used as a standard (Bio-Rad protein assay kit, Bio-Rad).

**RESULTS**

As shown in Figure 1, the diluted antiserum (1:250 000) recognized a single band of epididymal plasma that had the same electrophoretic mobility as the antiagglutinin. Additionally, no antigen of epididymal plasma reacted with the diluted preimmune serum (1:10 000).

The antiagglutinin was purified more effectively by the procedures described in *Materials and Methods* than by the procedure previously used [23]. More than 300 µg of the antiagglutinin was obtained from 10 ml of epididymal plasma in this study (data not shown).

As shown in Figure 2, a positive reaction of sialic acid residues was detected in the antiagglutinin. However, no reaction was observed in the samples treated chemically to remove sialic acid residues.

Figure 3 shows the reactivity of the antiagglutinin with the specific antiserum after the chemical treatment to remove sialic acid residues. In neither the antiagglutinin included in epididymal plasma nor the purified antiagglutinin was a reaction with the specific antiserum observed after the chemical treatment. Similarly, the reactivity of the antiagglutinin included in epididymal plasma with the specific

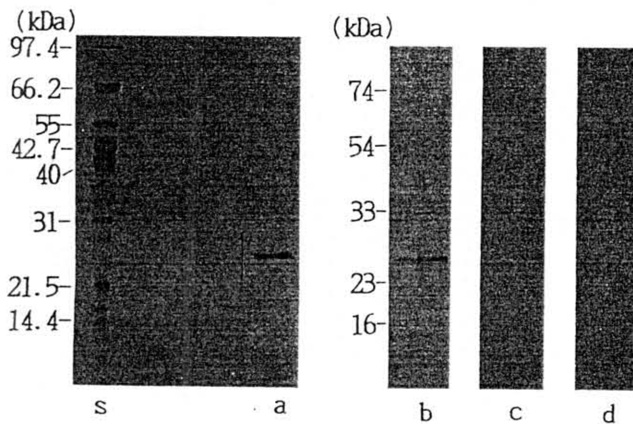



FIG. 2. Detection of sialic acid residues in antiagglutinin. Lane a: antiagglutinin (1.5 μg protein) silver-stained after SDS-PAGE. Lanes b–d: antiagglutinin (1.5 μg protein each) transblotted to PVDF membrane after SDS-PAGE and then used for detection of sialic acid residues. Lane b: incubated with 1 mM sodium metaperiodate. Lane c: incubated without sodium metaperiodate (control). Lane d: incubated with 1 mM sodium metaperiodate after chemical removal of sialic acid residues (control). Lane s: molecular mass standards.

antiserum was greatly reduced by the treatment with neuraminidase (Fig. 4).

As shown in Figure 5, the antiagglutinin was separated into one major (pI 5.8) and two minor forms (pI 5.6 and pI 5.95) in the two-dimensional PAGE pattern. A pattern corresponding to that of the purified antiagglutinin was also obtained in the Western blotting of epididymal plasma (Fig. 6). Furthermore, the major form of the antiagglutinin (pI 5.8) had affinity to the WGA-peroxidase reagent (Fig. 7). However, no binding of the WGA-peroxidase reagent to the minor forms was observed. Additionally, the preimmune serum (1:10 000) recognized no spot of epididymal plasma (Fig. 6).

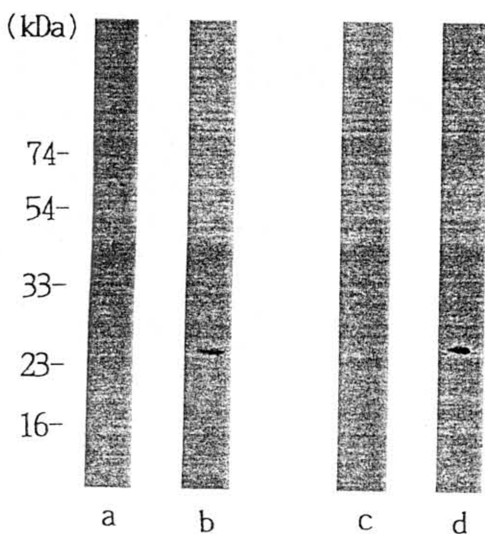



FIG. 3. Effects of chemical removal of sialic acid residues on immunoreactivity of antiagglutinin. Lanes a and b: epididymal plasma (5 μg protein each). Lanes c and d: purified antiagglutinin (1 μg protein each). Before immunodetection by antiserum (1:250 000), blotted membranes were treated in 25 mM $H_2SO_4$ for 1 h at 80°C and then washed three times (lanes a and c). In control experiments (lanes b and d), this treatment was omitted.

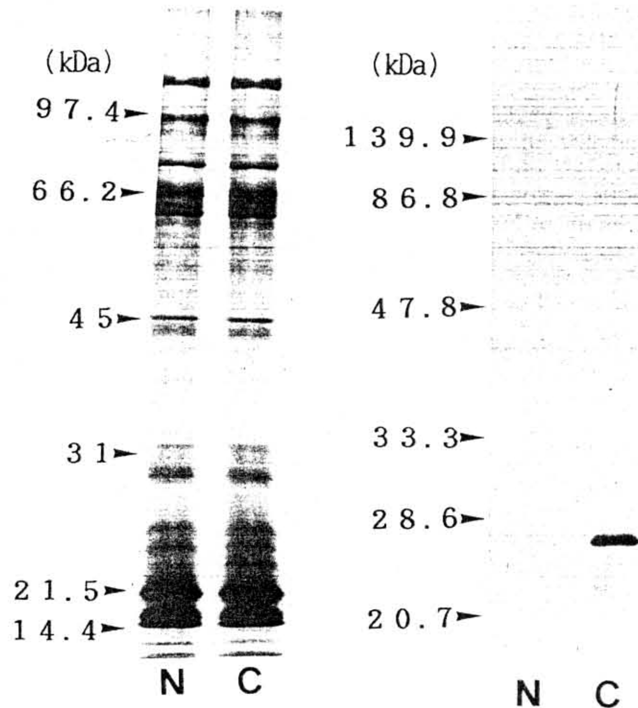



FIG. 4. Effects of enzymatic removal of sialic acid residues on immunoreactivity of antiagglutinin. Samples were epididymal plasma (5 μg protein each). Before SDS-PAGE, epididymal plasma was treated with neuraminidase (lane N) or with inactivated neuraminidase (lane C, control) for 20 h at 37°C. **Left** and **right** panels show patterns silver-stained and immunodetected by antiserum (1:250 000), respectively.

Figure 8 shows two-dimensional PAGE patterns of extracts from the cauda epididymal spermatozoa. In the silver-stained pattern, a spot of 25 kDa and pI 5.8 (the same electrophoretic mobility as the major form of the antiagglutinin) was observed, though no spot was detected of the same mobility as the minor forms. Moreover, the spot (25 kDa, pI 5.8) was recognized by the antiserum against the antiagglutinin.

As shown in Figure 9, the antiserum recognized a single band with almost the same mobility as the antiagglutinin in seminal plasma, although the reaction in seminal plasma was weaker than that in epididymal plasma. The single

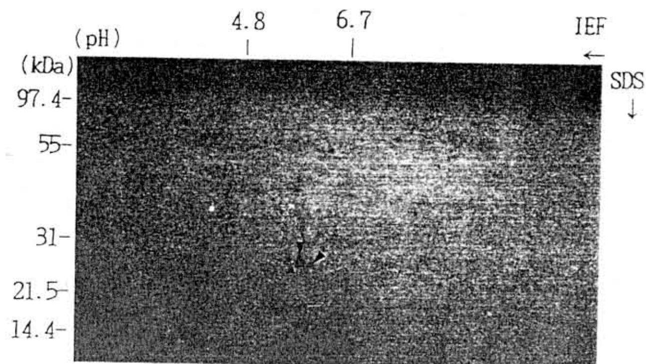



FIG. 5. Two-dimensional PAGE pattern of purified antiagglutinin (2 μg protein) silver-stained. Electrophoresis was performed with Multiphor II Electrophoresis System. Arrowheads indicate spots of antiagglutinin.

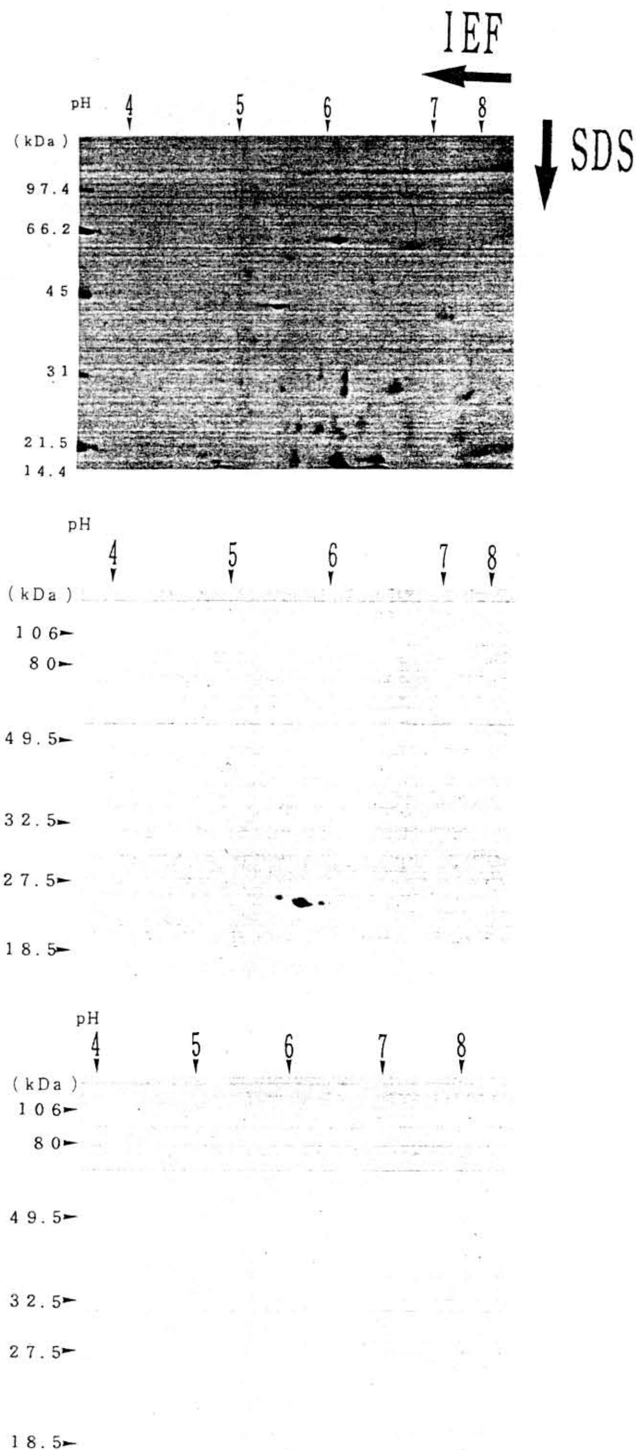



FIG. 6. Two-dimensional PAGE patterns of epididymal plasma (30 μg protein each), silver-stained (**top**), and immunodetected by antiserum (1:250 000; **center**) or preimmune serum (1:10 000; control; **bottom**). Electrophoresis was performed as described by O'Farrel [29], with minor modifications (see *Materials and Methods*).

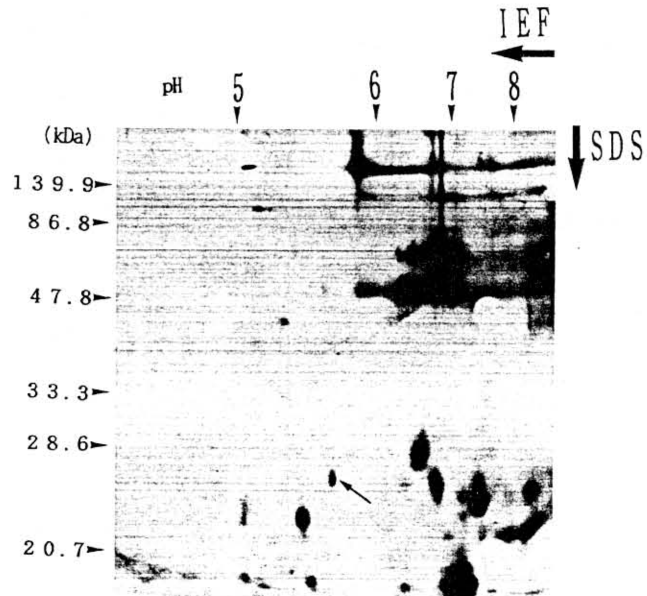



FIG. 7. Two-dimensional PAGE pattern of epididymal plasma (30 μg protein) detected by WGA-peroxidase reagent (0.5 μg protein/ml). Electrophoresis was performed as described by O'Farrel [29], with minor modifications (see *Materials and Methods*). Arrow indicates major spot of antiagglutinin.

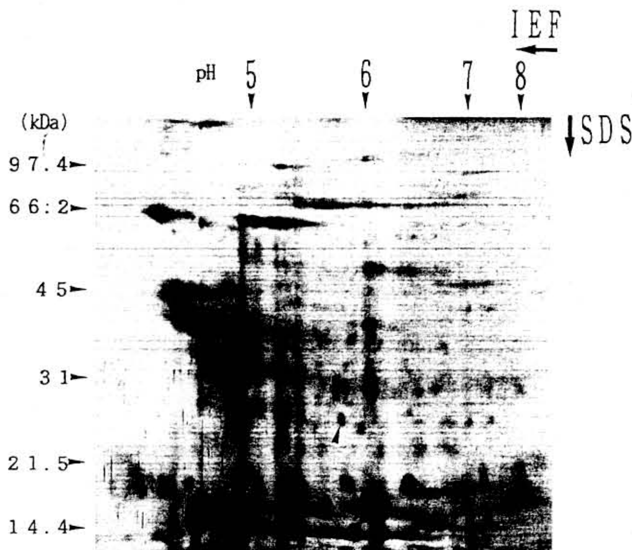
band was detected in extracts from ejaculated spermatozoa (Fig. 10). However, the band was not detected in extracts from spermatozoa after the incubation for capacitation (Fig. 10).

## DISCUSSION

Standard purification techniques, using the antiserum (see *Materials and Methods*) to monitor the presence of antiagglutinin, were used to prepare a purified preparation of the molecule(s) from boar epididymal plasma. The antiagglutinin, apparently homogenous in one-dimensional PAGE analysis, was separated (Figs. 5 and 6) into one major form (25 kDa; pI 5.8) and two minor forms after two-dimensional PAGE separation and detection with the antiserum.

Sialic acid residues linked to glycoproteins can be detected with WGA lectin, although that lectin also recognizes *N*-acetyl-D-glucosamine. Since only the major component of epididymal antiagglutinin (25 kDa; pI 5.8) reacted under those conditions (Fig. 7), we conclude that this molecular species, and not the minor components, is a sialic acid/*N*-acetyl-D-glucosamine-containing protein. That conclusion is further refined by the observation (Fig. 2) that preferential labeling of sialic acid residues by controlled oxidation [33] was eliminated by the chemical removal of sialic acids; this confirms the presence of sialic acid residues.

Many studies, including our results reported here (Figs. 3 and 4), have established the importance of sialic acid residues in antibody recognition. For example, in one case [34], sialic ~~acid~~ residues can mask the antigenic epitope of a sperm antigen (54-kDa rat sperm maturation sialoglycoprotein); removal of sialic acids exposes critical epitopes on the core protein. In another case [35, 36], the monoclonal antibody T305 antigen and surface antigens of the erythrocyte contain sialic acid residues that constitute the



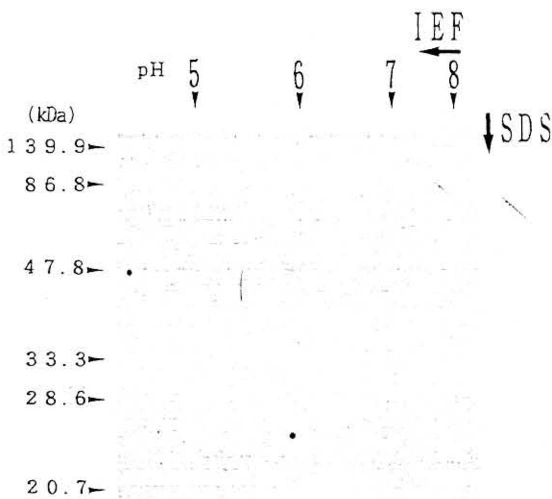




FIG. 8. Two-dimensional PAGE patterns of extracts from epididymal spermatozoa ($4.0 \times 10^7$ cells each), silver-stained (**top**) and immunodetected by antiserum (1:250 000; **bottom**). Electrophoresis was performed as described by O'Farrel [29], with minor modifications (see *Materials and Methods*). Arrowhead indicates the spot with the same mobility as the major spot of antiagglutinin.

unique epitope; removal of those residues destroys antibody recognition.

The observed molecular heterogeneity of the antiagglutinin is not unique. Similar molecular heterogeneity of male reproductive tract fluid proteins has been reported in bovine seminal plasma proteins [37] and a 135-kDa boar epididymal plasma protein [21]. Those examples of molecular heterogeneity appear to be due to the modifications after translation [37] and/or alternative splicing of mRNA [38]. Data for this protein suggest that posttranslational differences in sialylation and/or glycosylation may account for the microheterogeneity between the major and minor forms of the antiagglutinin.

A relationship between epididymal plasma protein and spermatozoal bound protein was established by the observation (Fig. 8) that the major form of epididymal plasma

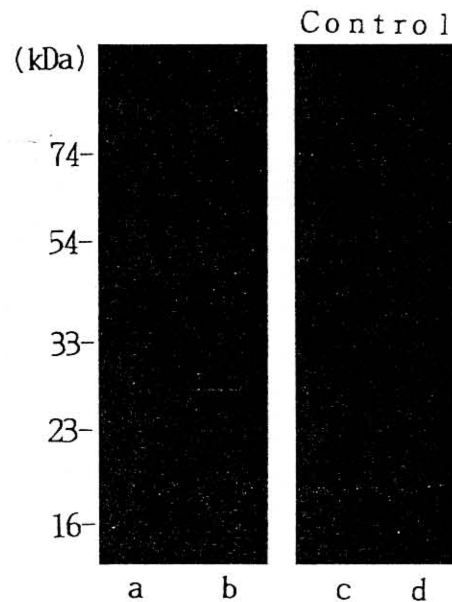



FIG. 9. SDS-PAGE of seminal (20 μg protein each, lanes a and c) and epididymal plasma (20 μg protein each, lanes b and d) immunodetected by antiserum (1:250 000; **left**) or preimmune serum (1:10 000; control; **right**).

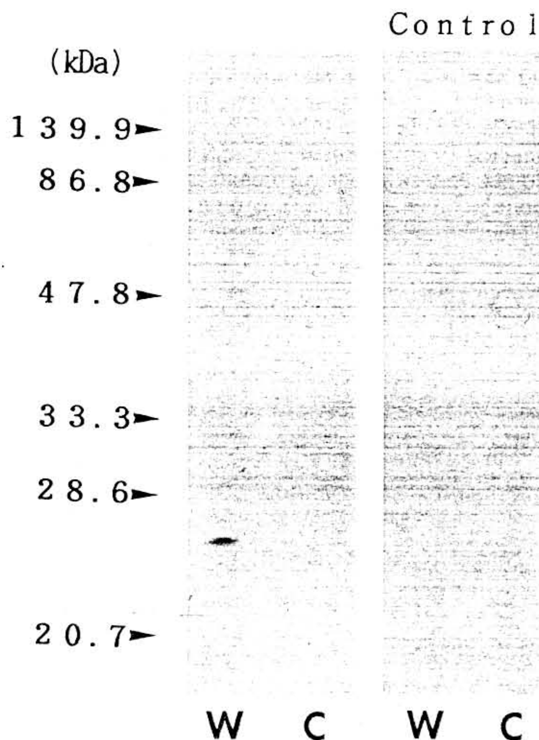



FIG. 10. SDS-PAGE of extracts from ejaculated spermatozoa ($8.0 \times 10^6$ cells each) immunodetected by antiserum (1:250 000; **left**) or preimmune serum (1:10 000; control; **right**). Samples used in the lanes marked **W** were washed spermatozoa and in the lanes marked **C** were spermatozoa incubated for capacitation in vitro after washing, respectively.

antiagglutinin was found in extracts from washed epididymal spermatozoa. Likewise, a cross-reacting material was found in seminal plasma (Fig. 9), with an apparently decreased concentration of the antiagglutinin at ejaculation due to factors such as dilution by secretions from accessory genital glands.

Evidence for changes in amounts of the cross-reacting material was also obtained in experiments (Fig. 10) testing for the effect of incubation under conditions known to induce in vitro capacitation. The cross-reacting material disappeared from ejaculated spermatozoa under these conditions, consistent with the suggestion of either loss of the antiagglutinin from the sperm surface or structural changes in the epitope of the antiagglutinin during the incubation. Additionally, it has been observed that most spermatozoa are agglutinated at the head with each other after incubation (personal communication of Dr. Kano, Kobe University).

Epididymal sialoproteins have been detected in a variety of species and are associated with maturation-dependent modifications of the sperm surface in the epididymis [39]. In rats, SP sialoprotein (37.5 kDa, pI 4.7) is secreted from the caput and corpus epididymides and binds to luminal spermatozoa [40]. Moreover, during transit through the epididymis, the amount of one 31-kDa sialoprotein on the spermatozoa increases, the amount of another (48-kDa) form remains constant, and amounts of several others decrease [41]. In mice, sperm maturation sialoprotein (called antigen 4 or T21 antigen) is secreted from the distal caput and proximal corpus epididymides and is absorbed by spermatozoa [39]. This antigen functions to prevent spermatozoa from tail-to-tail agglutination [42], and its sialic acid residues appear to be related to function since desialylation by neuraminidase treatment causes rapid sperm agglutination [34]. Our previous results [43] revealed that the antiserum against the antiagglutinin first reacts with the 25-kDa band of extracts from the corpus epididymal spermatozoa, and then reacts more strongly with the same band of extracts from the cauda epididymal spermatozoa. Taking these previous results together with results presented here, we suggest that the 25-kDa sialoprotein (antiagglutinin) gradually increases on luminal spermatozoa during epididymal maturation. This is supported by other reports that a 14–36-kDa sialoprotein family appears in boar spermatozoa during their transit through the epididymis [16].

Functions of sialic acids on the sperm surface still remain unclear. Holt [44] discussed the role of spermatozoal sialic acids in masking antigens of the sperm surface and keeping certain spermatozoa from phagocytosis by leukocytes and vaginal epithelial cells. In addition, it has been generally considered that sialic acid residues linked to glycoproteins are involved directly or indirectly in cell-to-cell interactions, including agglutination and disagglutination [35, 45]. It seems that the increase of the 25-kDa sialoprotein (antiagglutinin) on boar spermatozoa occurs in coincidence with reduction of the sperm agglutinability [1] and development of the sperm capacity to bind tightly to zona pellucida [3] and to egg plasma membrane [1]. Data for hamsters [46] are consistent with a conclusion that sperm surface sialoprotein appears to play an important role in sperm interaction with the epithelia of the isthmus of the oviduct. This part of the oviduct has been considered to be a sperm reservoir [47], from which spermatozoa are released, due to loss of or structural changes in the sperm surface sialoprotein that coincide with sperm capacitation, to reach the site of fertilization.

Since this antiagglutinin (sialoprotein) was lost from the sperm surface or changed in structure during sperm capacitation in vitro, we are very interested in the relationship between the antiagglutinin and sperm interaction with oviductal epithelia. Research directed toward elucidation of the relationships between the maturation-dependent appearance of the sialoprotein (antiagglutinin) on the spermatozoa and either sperm agglutinability or capacity to interact with an egg is also in progress.

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## REFERENCES


1. Dacheux JL, Paquignon M, Combarnous Y. Head-to-head agglutination of ram and boar epididymal spermatozoa and evidence for an epididymal antagglutinin. J Reprod Fertil 1983; 67:181–189.
2. Harayama H, Kato S. Changes in motility and morphology of spermatozoa during their transit through the epididymis in Meishan boars at various ages. Anim Sci Technol (Jpn) 1992; 63:462–467.
3. Peterson RN, Hunt WP, Henry LH. Interaction of boar spermatozoa with porcine oocytes: increase in proteins with high affinity for the zona pellucida during epididymal transit. Gamete Res 1986; 14:57–64.
4. Harayama H, Kusunoki H, Kato S. Capacity of rete testicular and cauda epididymal boar spermatozoa to undergo the acrosome reaction and subsequent fusion with egg plasma membrane. Mol Reprod Dev 1993; 35:62–68.
5. Holtz W, Smidt D. The fertilizing capacity of epididymal spermatozoa in the pig. J Reprod Fertil 1976; 46:227–229.
6. Hunter AG, Polge C. In vitro fertilization of spermatozoa from various regions of the boar epididymis. Biol Reprod 1986; 34(suppl):99 (abstract 100).
7. Hunter RHF, Holtz W, Henfrey PJ. Epididymal function in the boar in relation to the fertilizing ability of spermatozoa. J Reprod Fertil 1976; 46:463–466.
8. Hunter RHF, Holtz W, Herrmann H. Stabilizing role of epididymal plasma in relation to the capacitation time of boar spermatozoa. Anim Reprod Sci 1978; 1:161–166.
9. Eng LA, Oliphant G. Rabbit sperm reversible decapacitation by membrane stabilization with a highly purified glycoprotein from seminal plasma. Biol Reprod 1978; 19:1083–1094.
10. Reynolds AB, Thomas TS, Wilson WL, Oliphant G. Concentration of acrosome stabilizing factor (ASF) in rabbit epididymal fluid and species specifity of anti-ASF antibodies. Biol Reprod 1989; 40:673–680.
11. Bedford JM. Maturation, transit and fate of spermatozoa in the epididymis. In: Astwood EB, Greep RO (eds.), Handbook of Physiology. Washington, DC: American Physiological Society; 1975: 303–318.
12. Orgebin-Crist MC, Danzo BJ, Davies J. Endocrine control of the development and maintenance of sperm fertilization ability in the epididymis. In: Astwood EB, Greep RO (eds.), Handbook of Physiology. Washington, DC: American Physiological Society; 1975: 319–338.
13. Setchell BP, Maddocks S, Brooks DE. Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 2nd ed. New York: Raven Press, Ltd; 1994: 1063–1175.
14. Hammerstedt RH, Parks JE. Changes in sperm surface associated with epididymal transit. J Reprod Fertil Suppl 1987; 34:133–149.
15. Russell LD, Peterson RN, Hunt W, Strack LE. Posttesticular surface modifications and contributions of reproductive tract fluids to the surface polypeptide composition of boar spermatozoa. Biol Reprod 1984; 30:959–978.
16. Dacheux JL, Dacheux F, Paquignon M. Changes in sperm surface membrane and luminal protein fluid content during epididymal transit in the boar. Biol Reprod 1989; 40:635–651.
17. Bostwick EF, Bentley MD, Hunter AG, Hammer R. Identification of a surface glycoprotein on porcine spermatozoa and its alteration during epididymal maturation. Biol Reprod 1980; 23:161–169.
18. Dacheux F, Dacheux JL. The intracellular pathway of antagglutinin secretion in the boar caput epididymidis as revealed by immunogold labelling. Cell Tissue Res 1987; 249:89–99.
19. Dacheux F, Dacheux JL. Immunocytochemical localization of antagglutinin in the boar epididymis. Cell Tissue Res 1988; 252:329–337.