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SHORT COMMUNICATION

Activity of exoglycosidases in ejaculated spermatozoa of boar and bull

Masayuki Hayashi[1], *Naoto Yonezawa*[1,2], *Toshiyuki Katsumata*[3], *Keiichi Ikeda*[1], *Fabiana Lica Imai*[1], *Kazuhiro Kikuchi*[4], *Seizo Hamano*[5] *and Minoru Nakano*[1,2]

Graduate School of Science and Technology and Department of Chemistry, Chiba University, Chiba; Tokyo Medical and Dental University, Ichikawa; National Institute of Agrobiological Resources, Tsukuba; and Livestock Improvement Association, Tokyo, Japan

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Summary

The activity of exoglycosidases in extracts from freshly ejaculated boar and bull spermatozoa with 0.2% Brij-35/2% acetic acid was measured. The results show that β-*N*-acetylhexosaminidase, β-galactosidase and α-mannosidase are the major glycosidases; much higher levels of activity were found in boar spermatozoa than in bull spermatozoa. When compared on a per spermatozoon basis, the ratios of the activities of β-*N*-acetylhexosaminidase, β-galactosidase and α-mannosidase in boar spermatozoon relative to those in bull spermatozoon were approximately 13 000:1, 1700:1 and 400:1, respectively. Liberation of these glycosidases from bull spermatozoa by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) was low, in contrast to liberation of α-mannosidase from boar spermatozoa previously found by the same means. The possibility that the exoglycosidases present in large amounts in boar spermatozoa play a role in the process of binding to the zona pellucida glycoprotein of the egg is discussed.

Keywords: Acrosome reaction, GPI anchor, Sperm glycosidase, Sperm–egg recognition, Zona pellucida

Introduction

In the early phase of mammalian fertilization, a spermatozoon binds to the zona pellucida, the transparent envelope of oocyte. This initial binding is achieved in a species-specific manner, and although many candidates for complementary molecules of both gametes of various animal species have been proposed, *bona fide* ligands have not been fully characterized (Litscher & Wassarman, 1993; Tulsiani *et al*., 1997; Benoff, 1997; Töpfer-Petersen, 1999).

The egg zona is composed of three glycoprotein components – ZPA, ZPB and ZPC (Harris *et al*., 1994) – and spermatozoa bind to the carbohydrate moieties of one of these zona glycoproteins (Litscher & Wassarman, 1993; Prasad *et al*., 2000). In the mouse, α-galactose (Bleil & Wassarman, 1988), β-*N*-acetylglucosamine (Miller *et al*., 1992) or α-fucose (Johnston *et al*., 1998), all found at the nonreducing end of O-linked chains of ZPC (also known as ZP3), or α-mannose (Cornwall *et al*., 1991), found on N-linked chains of ZPC, have been reported to be sperm ligands, but a recent report on the carbohydrate chain structures does not support any of these residues, with the exception of α-mannose (Easton *et al*., 2000). In the pig, triantennary and tetra-antennary complex-type chains of ZPB have been suggested to be the major sperm ligand, based on evidence from competition assays,

All correspondence to: Dr M. Nakano, Department of Chemistry, Faculty of Science, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan. Tel: +81 43 290 2794. Fax: +81 43 290 2874. e-mail: mnakano@faculty.chiba-u.jp
[1] Graduate School of Science and Technology, Chiba University, Chiba, 263-8522, Japan.
[2] Department of Chemistry, Faculty of Science, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba, 263-8522, Japan.
[3] College of Liberal Arts and Science, Tokyo Medical and Dental University, Ichikawa, 272-0827, Japan.
[4] Department of Genetic Resources II, National Institute of Agrobiological Resources, Tsukuba, 305-8602, Japan.
[5] Animal Bio-Technology Center, Livestock Improvement Association, Tokyo, 140-0002, Japan.

but the inhibition of sperm–zona binding by these chains is incomplete (Kudo *et al*., 1998). Recently, the *N*-acetyllactosamine region in the N-linked carbohydrate chains of pig zona proteins has been suggested to be involved in sperm recognition (Dunbar *et al*., 2001). It has also been shown by two competition assay methods that the bovine sperm ligand is α-mannosyl residues present at the non-reducing ends of high-mannose-type chains of the bovine zona proteins, although the responsible component in the zona is still unclear (Amari *et al*., 2001).

Since the sperm ligands are carbohydrate chains of the zona proteins, the complementary binding molecule of the spermatozoon should possess lectin-like properties. Accordingly, glycosyltransferases and glycosidases in spermatozoa may be responsible for binding to the zona. In fact, sialyltransferase, fucosyltransferase and β1,4-galactosyltransferase in mouse (Durr *et al*., 1977; Ram *et al*., 1989; Miller *et al*., 1992); α-mannosidase in mouse (Cornwall *et al*., 1991), rat (Tulsiani *et al*., 1989) and human (Tulsiani *et al*., 1990); and β-*N*-acetylhexosaminidase in human (Miranda *et al*., 2000) have each been proposed as candidates for the complementary molecule of spermatozoa.

In the present study, we measured exoglycosidase activities in freshly ejaculated boar and bull spermatozoa in which the glycosidases responsible for recognition of the zona have not yet been demonstrated.

Materials and methods

Materials

4-Methylumbelliferyl-β-galactoside (4MU-β-Gal), 4-methylumbelliferyl-α-mannoside (4MU-α-Man), 4-methylumbelliferyl-β-mannoside (4MU-β-Man), 4-methylumbelliferyl-β-*N*-acetylgalactosaminide (4MU-β-GalNAc), 4-methylumbelliferyl-β-*N*-acetylglucosaminide (4MU-β-GlcNAc), 4-methylumbelliferyl-α-*N*-acetylneuraminide (4MU-α-NeuNAc) and phosphatidylinositol-specific phospholipase C (PI-PLC) were purchased from Sigma.

Preparation of the acid extracts from spermatozoa

All experimental procedures were performed at 4 °C unless otherwise stated. Freshly ejaculated boar semen (100 ml) was centrifuged at 900 *g* for 10 min to remove seminal plasma. Sperm pellets were washed eight times by suspension in 100 ml of phosphate-buffered saline (PBS) and centrifugation at 900 *g* for 10 min. The washed spermatozoa were suspended in 50 ml of 0.2% Brij-35/2% acetic acid and the suspension was homogenized with a Teflon homogenizer. The homogenized suspension was centrifuged at 100 000 *g* for 30 min. This extraction procedure was repeated twice and the extracts were combined.

Bull spermatozoa were similarly prepared from freshly ejaculated semen (0.5 ml) by suspension in 0.5 ml of PBS and centrifugation at 600 *g* for 10 min. The supernatants collected from two extractions, each performed with 0.5 ml of 0.2% Brij-35/2% acetic acid and centrifugation at 100 000 *g* for 30 min, were combined.

Glycosidase activities of the acid extracts

The exoglycosidase activities of the acid extracts were quantitated by measuring the liberation of 4-methylumbelliferone (4MU) from respective enzyme-specific substrates. The substrate (0.05 M in 0.1 M sodium citrate, pH 4.8) was incubated with the acid extracts at 37 °C for 30 min, and the reaction was stopped by addition of 0.4 M glycine-NaOH, pH 10.5. The fluorescence intensity of the mixture relative to that of standard 4MU (10 μM) was measured using excitation and emission wavelengths of 368 and 449 nm, respectively. The concentration of spermatozoa was determined using a haemocytometer and 1 unit of activity is defined as the activity that releases 1 μmol of 4MU per minute.

Digestion of washed bull spermatozoa with PI-PLC

Washed bull spermatozoa from 0.2 ml of ejaculated semen were suspended in 1.1 ml of PBS; 0.5 ml aliquots of the suspension were submitted either to PI-PLC digestion or to acid extraction. About 10^8 spermatozoa were incubated with 1.0 U of PI-PLC in 0.5 ml of PBS, pH 7.4, at 37 °C for 30 min. After centrifugation at 900 *g* for 10 min, the glycosidase activities of an aliquot of supernatant corresponding to 10^7 spermatozoa were assayed. Alternatively, 0.5 ml of the washed spermatozoa suspension was centrifuged at 900 *g* for 10 min. The pellets were submitted to the acid extraction procedure, followed by assay of glycosidase activity, as described above.

Results and discussion

Exoglycosidase activities in the acid extracts of boar and bull spermatozoa

Washing of boar and bull spermatozoa eight times, by repeated suspension in PBS and centrifugation, completely removed the glycosidases present in the seminal plasma. When the washed spermatozoa were homogenized with 0.2% Brij-35/2% acetic acid, several glycosidases were released into the supernatant. Since the enzyme possessing β-*N*-acetylglucosamidase activity in spermatozoa from both species also

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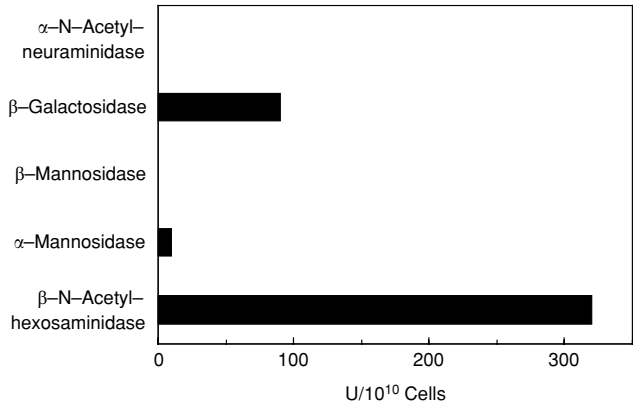

Figure 1 Exoglycosidase activities in the acid extract from boar spermatozoa with 0.2% Brij-35/2% acetic acid. β-*N*-Acetylhexosaminidase activity is expressed by β-*N*-acetylglucosaminidase activity. Duplicate experiments gave results within 5% error.

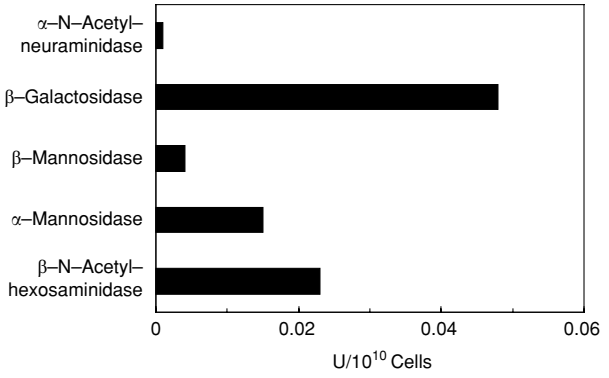

Figure 2 Exoglycosidase activities in the acid extract from bull spermatozoa with 0.2% Brij-35/2% acetic acid. β-*N*-Acetylhexosaminidase activity is expressed by β-*N*-acetylglucosaminidase activity. Duplicate experiments gave results within 5% error.

hydrolysed β-*N*-acetylgalactosaminide, it was termed β-*N*-acetylhexosaminidase. Of the exoglycosidases measured, β-*N*-acetylhexosaminidase was the most abundant enzyme in the acid extracts of ejaculated boar spermatozoa (Fig. 1). Significant amounts of β-galactosidase and α-mannosidase were also found, but α-*N*-acetylneuraminidase and β-mannosidase were barely detected. In the bull spermatozoa, the most abundant exoglycosidase was β-galactosidase (Fig. 2). Significant amounts of α-mannosidase and β-*N*-acetylhexosaminidase were present, but the amounts of β-mannosidase and α-*N*-acetylneuraminidase were quite low, when detectable at all. The amounts of glycosidases in boar and bull spermatozoa differed considerably from each other. The ratios of activities of β-*N*-acetylhexosaminidase, β-galactosidase and α-mannosidase found in boar spermatozoon relative to the activities in bull spermatozoon were approximately 13 000:1, 1700:1 and 400:1, respectively.

Release of exoglycosidases with PI-PLC from washed spermatozoa

We have previously shown that about half the α-mannosidase activity in acid extracts of boar spermatozoa arises from glycosylphosphatidylinositol (GPI)-anchored enzyme (Kuno *et al*., 2000). In the present study, to investigate the form in which the glycosidases are present, the activities of three exoglycosidases released by treating washed spermatozoa with PI-PLC were measured. Table 1 shows the ratios of the enzyme activities released by PI-PLC treatment relative to the activity of each enzyme found in the acid extracts. The presence of the glycosidases having GPI-anchored forms in bull spermatozoa could not be ascertained, since the amount of ejaculated bull semen was limited. The possibility that small amounts of the measured glycosidases in bull spermatozoa have GPI anchors cannot be ruled out, however. Incidentally, β-*N*-acetylhexosaminidase activity, which was not measured in the previous work (Kuno *et al*., 2000), could not be detected at all when boar spermatozoa were treated with PI-PLC in the current experiments. That is, the β-*N*-acetylhexosaminidase activity ratio of PI-PLC extract to acid extract was 0.

Function of glycosidases in recognition of the zona

To clarify the function of glycosyltransferase and glycosidase, their localization in spermatozoa should

Table 1 Release of glycosidases from ejaculated bull spermatozoa (10^7 cells) by treatment with PI-PLC/PBS and with Brij-35/acetic acid

	α-Mannosidase	β-Galactosidase	β-*N*-Acetylhexosaminidase
A: PI-PLC/PBS	1.34×10^{-6} U	5.35×10^{-6} U	1.34×10^{-6} U
B: 0.2% Brij-35/ 2% acetic acid	1.41×10^{-5} U	4.63×10^{-5} U	2.18×10^{-5} U
A/B	0.10	0.12	0.06

Duplicate experiments gave almost identical results.
PBS, phosphate-buffered saline.

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be considered. In murine spermatozoa, β1,4-galactosyltransferase has been shown to be present in the plasma membrane (Miller *et al*., 1992), but this enzyme is absent from human sperm plasma membrane (Tulsiani *et al*., 1990). Since several exoglycosidases are present in the acrosome, the initiation site of the acrosome reaction should also be taken into account, in order to clarify the function of acrosomal glycosidases. In the mouse and human, acrosome-intact spermatozoa bind to the zona, followed by induction of the acrosome reaction (Wassarman, 1988; Liu & Baker, 1990), while in guinea pigs completely acrosome-reacted spermatozoa bind to the zona (Yanagimachi, 1981). Furthermore, partially acrosome-reacted spermatozoa have been shown to bind to the zona in the pig (Jones *et al*., 1988; Takada *et al*., 1994; Yoshizawa *et al*., 1994; Yonezawa *et al*., 1995). In general, maximal activities of exoglycosidases are exhibited in acidic conditions. Therefore, the sperm exoglycosidases may play a role not in hydrolysis, but rather in recognition of carbohydrates of the zona glycoproteins. Nevertheless, their hydrolysing activity should also be considered, since several sperm exoglycosidases exhibit their activity even at neutral pH, although not optimally. When the acrosome reaction occurs after binding to the zona, the acrosomal enzymes might be responsible for penetration of a spermatozoon through the zona. When the acrosome reaction occurs before the spermatozoon reaches the zona, the acrosomal enzymes play a role in cumulus dispersion. The three main exoglycosidases in boar spermatozoon were 2 to 4 orders of magnitude more active than those in bull spermatozoon. When an acrosome-intact or partially acrosome-reacted boar spermatozoon binds to the zona at fertilization, these large amounts of exoglycosidases may hydrolyse the non-reducing terminal carbohydrate residues of the zona glycoproteins during the binding process.

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