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# *N*-Acetyl β-*D*-glucosaminidase is not attached to human sperm membranes through the glycosylphosphatidyl inositol (GPI)-anchor

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**Keywords:** *N*-acetyl β-*D*-glucosaminidase (NAGA); cell membrane; microsomes; alkaline phosphatase; adenosine triphosphatase; GPI anchor; PI-PLC

**Abstract** **Aim:** The mode of anchorage of *N*-acetyl β-*D*-glucosaminidase (NAGA) on human ejaculated sperm was investigated. **Methods:** Sperm plasma membrane was prepared by discontinuous sucrose gradient centrifugation from human sperm. NAGA was solubilized from these membranes by two detergents: octyl-glycoside and triton X-100. In separate studies, the release of the enzyme from the sperm membrane preparation by phosphatidylinositol specific phospholipase C (PI-PLC) was also examined. **Results:** NAGA activity was detected on sperm membranes isolated from human ejaculates. The pattern of the enzyme solubilization by detergents indicated that the enzyme was an integral protein of sperm membrane. NAGA was not released from the sperm membranes by PI-PLC treatment. **Conclusion:** The evidence presented strongly suggests that human sperm membrane bound NAGA is not attached via the GPI anchor. *( Asian J Androl 2002 Mar; 4 : 27-33 )*

## 1 Introduction

The role of carbohydrates in mammalian fertilization and related events has been extensively reviewed in recent times [1-3]. Glycosidases are an important group of enzymes responsible for cleaving specific sugar residues from glycoconjugates thereby facilitating modification to the surface of gametes that result in better sperm-egg interaction [4]. The male reproductive tract is one of the richest sources of *N*-acetylglucosaminidase (NAGA) and there is substantial evidence to suggest that this enzyme is copiously secreted from the epididymal epithelial cells [5,6]. Despite their abundance in the luminal fluid it is unlikely that the secreted form of this enzyme act to modify the sperm surface since the optimal pH for activity is acidic, a condition which is rarely encountered in the male reproductive tract. Besides, its natural substrate *N*-acetyl β-*D*-glucosamine residues are found to increase rather than decrease on the sperm surface as they move from the caput to the cauda epididymis [7]. Instead, it is believed that the extraordinarily high levels of NAGA found in the fluids surrounding the sperm, prevents the initiation of premature acrosomal reaction by

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binding to glucosamine that triggers this process [8].

Studies of NAGA from several sources have convincingly shown that the enzyme is an oligomeric protein and that the characteristics of the enzyme are governed by the composition of its subunits [9]. NAGA is known to exist as isozymes in both soluble and membrane-bound forms. There is overwhelming support to the contention that NAGA is carried on the surface of sperm for action at a later time during interaction with the egg [10]. NAGA is suggested to block polyspermy by cleaving and releasing glucosamine residues on zona antigens following fertilization [11]. Although the enzyme released from the cortical granules after fertilization may be largely responsible for the block to polyspermy, it is suspected that the enzyme carried on the sperm surface may participate in the process as well. It has been suggested that NAGA is incorporated into sperm during passage through the epididymis [7]. In order to decipher the molecular mechanisms by which sperm membranes acquire NAGA it would be first desirable to know how they are anchored on the sperm surface.

Compared to all the different kinds of membrane attachments known for sperm proteins, the GPI-anchor offers special structural and functional advantages. The distribution/translocation of this class of protein on the sperm during development, maturation, storage, capacitation, sperm-egg interaction and the acrosomal reaction clearly demonstrates their importance in reproduction [12]. GPI-anchored proteins are capable of unhindered lateral mobility on the membrane and are now known to be a part of a unique signal transduction mechanism [13]. In this paper, we have examined if NAGA from membranes of human ejaculated sperm is GPI anchored.

## 2 Materials and methods

### *2.1 Materials*

Human semen samples were collected from 12 healthy volunteers (age group 28-45 years) with proven fertility. After 30 minutes of liquifaction at room temperature, only the samples that showed normal sperm density, motility and morphology were included in this study. Whenever more samples were required they were obtained from among these donors following at least three days of sexual abstinence. *p*-Nitrophenyl-*N*-acetyl β-*D*-glucosaminide was from Sigma chemical company. *p*-Nitrophenyl phosphate disodium salt was obtained from SRL (India). All other chemicals were of analytical grade and were purchased from E. Merck (India) Ltd., SD Fine chemicals and Rankem. Triton X-100 was from SRL, India and octyl glycoside was from Sigma chemical company, USA.

### *2.2 Purification of PI-PLC*

PI-PLC was isolated from *Bacillus cereus* MTCC 430 grown in our laboratory. The enzyme purification protocol adopted was exactly the same as previously described [14]. One unit corresponds to amount of enzyme, which hydrolyzes 1 μmol 3-*sn*-phosphatidylinositol per min at pH 7.5 and 37 ¡æ

### *2.3 Isolation of sperm and preparation of plasma membrane*

The procedure used was that described earlier for human sperm [15]. Briefly, semen was allowed to liquefy at room temperature for 45 min, diluted with 5 volumes of ice-cold PBS and centrifuged at 600×*g* for 20 min at 4 ¡æ The pellet containing predominantly spermatozoa was suspended in ice-cold PBS (one-half original semen volume), layered on 11% dextran and centrifuged again at 600×*g* at 4 ¡æfor 20 min. Spermatozoa free from other seminal plasma components settled at the bottom. The dextran washed sperm was suspended in 5 mL of ice cold PBS and subjected to nitrogen cavitation (600 psi, 10 min equilibration). Sperm plasma membrane (SPM) was prepared from the cavitated sample by discontinuous sucrose gradient centrifugation.

### *2.4 Preparation of microsomes*

Microsomes were prepared from goat kidney by a previously published procedure [16]. Briefly, goat kidney was obtained from the local abattoir within 1h of killing the animal. The tissue was cut into thin slices and homogenized (sucrose 0.25 mol/L, 9 mL/g tissue) in a glass homogeniser with a Teflon pestle (1000 rpm, 12 up and down strokes). The homogenate was centrifuged at 15, 000×*g* for 20 min in a J 21 Beckman high-speed centrifuge. The supernatant obtained was re-centrifuged at 105,000×*g* for 60 min in a Beckman ultracentrifuge LS-50B: Ti 50 rotor. The resulting pellet was taken as the microsomal fraction (MF).

### *2.5 Cold solubilization of membrane bound enzymes*

Proteins from SPM were solubilized using two detergents: triton X-100 (TX-100) and octyl glycoside (OG). Briefly, the SPM was incubated at 0.1%, 0.2% and 0.3% of each detergent on ice for 30 min. During the period of incubation, the samples were vortexed every 10 min and returned to the ice bath. The mixture was then centrifuged at 105,000×*g* at 4 ¡æfor 30 min and the resulting supernatant was assayed for enzyme activity and protein concentration. For comparison purposes, membrane bound alkaline phosphatase (AP) and adenosine triphosphatase (ATPase) was also solubilized with the same detergents from MF.

*2.6 Cleavage by PI-PLC treatment*

Equal aliquots of ejaculated whole sperm ($4\times10^7$ cells) were incubated with and without PI-PLC (2 units/tube) at 37 ¡æto monitor the release of NAGA from sperm. After 2 h, the incubation mixture was centrifuged at 10,000×*g* for 20 min in a Sigma 15K refrigerated centrifuge and the NAGA activity measured in the supernatant and pellet fractions.

A similar protocol was followed with membrane preparations. The original activity of NAGA in SPM and AP/ATPase in MF were estimated as per methods detailed under enzyme assays. Aliquots of the MF and SPM were then incubated with and without PI-PLC (2 units/tube). Incubations were carried out at 37 ¡æfor 2 hours. At the end of the incubation period, the reaction mixture was centrifuged at 105,000×*g* for 60 min and the activity of the respective membrane bound enzymes was measured in both the pellet and supernatant.

*2.7 Positive and negative controls*

Two microsomal enzymes, with and without the GPI anchors were used as positive and negative controls. AP is a GPI anchored protein, which is released from the microsomal fraction of hepatic and kidney tissues by PI-PLC treatment. The other membrane-bound enzyme ATPase is an integral protein but is not attached by the GPI anchor.

*2.8 Enzyme assays*

In preliminary investigations, we found that sperm-bound NAGA showed maximum activity at near neutral pH and therefore NAGA activity was measured at pH 6.0. The standard incubation mixture (0.5 mL) contained 5mmol/L *p*-nitrophenyl β-*D*-glucosaminide, an aliquot of the sample and 0.1mol/L $KH_2PO_4$-NaOH buffer, pH 6.0. After incubation for the desired period at 37 ¡æ, the reaction was arrested by the addition of 1 mL alkaline buffer (0.133 mol/L glycine, 0.083 mol/L $Na_2CO_3$ and 0.067mol/L NaCl, pH 10.7). The amount of *p*-nitrophenol released was quantified by measuring the absorbance at 400 nm. AP activity was measured using *p*-nitrophenyl phosphate as substrate [16]. In both cases, one unit is defined as the amount of enzyme that catalyses the release of 1 μmol PNP/h. The activity of ATPase was estimated by measuring the amount of phosphate released by the enzyme [17]. One unit denotes the amount of enzyme that catalyses the release of 1 μmol phosphate/h.

Protein was measured by the Pierce BCA protein assay kit (Product No.23225) according to protocol outlined in manufacturer's instructions using bovine serum albumin as the standard.

Statistical analysis was done by the Student's *t*-test.

## 3 Results

The semen samples collected from healthy volunteers showed the following characteristics: 4.7-6.2 mL volume, pH 7.0-8.3 , 16-25 min liquifaction time, > $12\times10^7$ total cell counts, 80-91% vitality, class "a" rapid progressive motility and 71-91% acrosome intact sperm. Thus, all the parameters measured conformed to the WHO standard criteria used for surveillance of normal semen. Moreover, the individuals from whom samples were obtained had proved fertility within the last four years.

The PI-PLC used in the experiments were prepared from the culture medium in which *Bacillus cereus* was grown under conditions specified by the supplier of the culture. The culture medium was subjected to sequential purification steps detailed in Table 1. The final enzyme was purified 58 fold and had an activity of 482 U/ mg protein.

Table 1. Purification of PI-PLC from *Bacillus cereus* MTCC 430.

| Step | Total protein (mg) | Total activity (U) | Specific activity | Purification (fold) | Recovery (%) |
|---|---|---|---|---|---|
| Culture medium | 80 | 666 | 8.32 | 1 | 100 |
| 60%-satd.-$(NH_4)_2SO_4$ precipitation | 22 | 490 | 21.80 | 2.62 | 74 |
| DEAE cellulose | 1.3 | 320 | 246.10 | 29.60 | 48 |
| CM cellulose | 0.6 | 289 | 481.60 | 57.80 | 43 |

NAGA is known to exist in a soluble form within the acrosome of sperm and also in a form bound to its plasma membrane. We wanted to first test if the NAGA bound to sperm membranes could be released from the sperm. Therefore, we treated whole sperm with PI-PLC and then centrifuged the sample to measure the released NAGA in the supernatant (Figure 1). Even though NAGA activity was predominantly confined to the pellet, the activity in supernatant was not low enough to be ignored. There was only a 4-7 % increase in the number of acrosome-reacted sperm after the treatment implying that NAGA activity in the supernatants of sperm samples with and without PI-PLC treatment may be due to a loosely adsorbed isoform of the enzyme released during the incubation and centrifugation procedure. Besides, the results also demonstrate that membrane bound NAGA is not released by PI-PLC treatment and therefore its association with the membrane is not likely to be through the

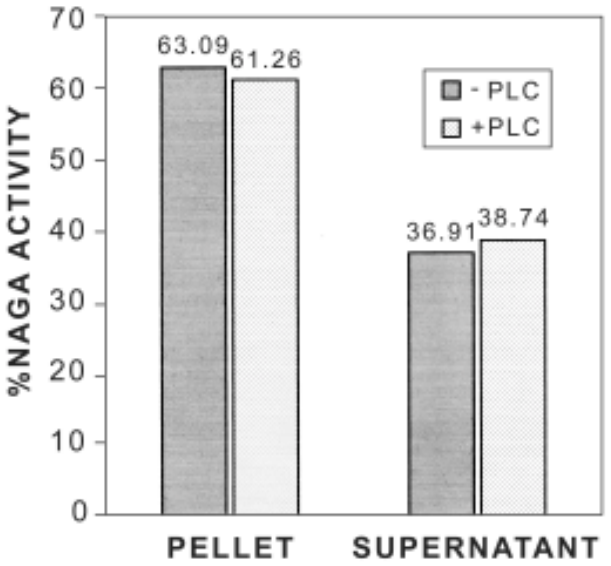


Figure 1. Pattern of release of NAGA from whole sperm after PI-PLC treatment. Equal aliquots of ejaculated sperm ($4\times10^7$ cells) were incubated with and without PI-PLC at 37°C for 2 h. The mixture was then centrifuged at 10,000× *g* for 20 min and NAGA measured in both the supernatant and pellet fractions. No significant difference in NAGA activity between the two supernatants was observed.

GPI anchor. However, we needed to confirm that the PI-PLC used was potent and had not lost its activity. Therefore, we inducted a positive (AP) and negative (ATPase) control, both enzymes found on microsomal membranes along with studies on NAGA in subsequent experiments.

Membrane bound enzymes have been solubilized in a variety of detergents. We specifically chose the two detergents OG and TX-100 since GPI anchored proteins are better solubilised in OG than in TX-100 (Table 2). This is clearly reflected in the OG/TX-100 ratio of solubilization of proteins (Figure 2). In the present studies, as expected, the positive control (AP) was better solubilized in OG than TX-100 while the reverse was true of the negative control (ATPase) and sperm NAGA activity. Detection of enzyme activity in purified membrane preparations is a direct evidence for their strong association with membrane structure. Besides, it helps in ruling out the interference from soluble / loosely adsorbed forms of the same enzyme contained in whole cell preparations. Therefore investigations were carried out in MF and SPM and the pattern of enzymes released by the action of PI-PLC was analyzed (Table 3).

From the results presented it is pretty obvious that the activity of NAGA was not released into the supernatant fraction from SPM by PI-PLC, just as ATPase was not released from MF. It is therefore highly unlikely that

Table 2: Detergent solubilization of membrane bound enzymes.

| Detergent solubilization | | MF - ATPase | MF - AP | SPM- NAGA |
|---|---|---|---|---|
| OG | 0.1% | 13.4 ± 0.8 | 73 ± 4 | 0.409 ± 0.016 |
| | 0.2% | 8.6 ± 0.5 | 70 ± 3 | 0.325 ± 0.012 |
| | 0.3% | 7.9 ± 0.4 | 69 ± 5 | 0.157 ± 0.008 |
| TX-100 | 0.1% | 16.8 ± 1.0 | 62 ± 6 | 0.431 ± 0.020 |
| | 0.2% | 12.8 ± 0.7 | 57 ± 4 | 0.363 ± 0.018 |
| | 0.3% | 11.6 ± 0.6 | 54 ± 4 | 0.248 ± 0.013 |

The specific activity of the three enzymes after cold solubilization by the respective detergents is tabulated ($n$=3). MF: microsomal fraction from goat kidney; SPM: sperm plasma membrane from human ejaculates; AP: alkaline phosphatase; ATPase: adenosine triphosphatase; NAGA: *N*-acetyl β-*D*-glucosaminidase.

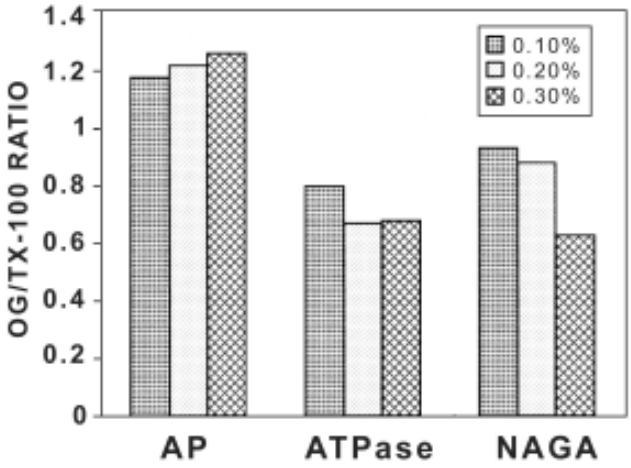


Figure 2. OG / TX-100 solubilization ratio of membrane bound enzymes. Isolated membrane fractions were subjected to cold solubilization using detergents at 0.1, 0.2 and 0.3 %. Enzymes showing values greater than 1 are supposed to be GPI-anchored. (OG, octylglycoside; TX-100, triton X-100)

sperm membrane associated NAGA is GPI anchored. The decrease in the AP activity of the pellet fraction in PI-PLC treated MF compared to the untreated control strongly suggests that AP was released by PI-PLC treatment as expected for GPI anchored proteins. This also implies that the PI-PLC used as a tool for cleavage was potent and could release proteins from their GPI anchors. One more interesting fact that came to light from these studies is that the activity of AP in the supernatant of PI-PLC treated MF was substantially higher than even the total activity of AP in the membrane preparation before digestion with PI-PLC. We checked our PI-PLC preparation and found no trace of any AP activity thus ruling out the possibility that the AP detected in the supernatant was due to contamination. We suspect that the activity of membrane-bound AP increases when the enzyme is re-

Table 3: Pattern of release of ecto-enzymes from membrane preprations after PI-PLC treatment.

| Enzyme | Total enzyme activity in membrane | Control (-PI-PLC) | | Test (+PI-PLC) | |
|---|---|---|---|---|---|
| | | Pellet | Supernatant | Pellet | Supernatant |
| Microsomal alkaline phosphatase | 95.2 ± 6.5 | 83.1 ± 4.6 | 1.98 ± 0.88 | 69.2 ± 4.0[c] | 100.0 ± 5.2[c] |
| Microsomal adenosine triphosphatase | 46.4 ± 3.0 | 40.7± 2.5 | 3.07 ± 1.01 | 42.7± 3.7 | 4.9 ± 1.3 |
| Sperm membrane NAGA | 14.6 ± 1.0 | 11.9 ± 0.9 | 1.16± 0.80 | 12.9± 1.4 | 2.05± 1.02 |

Microsomal and sperm plasma membranes were prepared as described in materials and methods. These membranes were divided into three equal aliquots. Total enzyme activity was measured in one aliquot and the remaining two were incubated in appropriate buffer with and without bacterial PI-PLC at 37°C for 2h. The enzyme released from the membranes were quantified in the pellet and supernatant ($n$=3) after centrifuging the incubation mixture at 105,000×$g$. [c] $P< 0.01$. Values obtained for the pellet/supernatant fraction are compared with the corresponding controls.

leased from the membrane. Perhaps the lipid environment of membranes suppresses AP activity and may be an important mechanism involved in the regulation of enzyme activity.

## 4 Discussion

NAGA is an active glycosidic enzyme found in sperm and its surrounding fluid [15]. Their action is of significance in reproductive processes since they have been implicated in a variety of functions ranging from prevention of premature acrosomal reaction [8] to blocking polyspermy [11]. There is now increasing evidence to suggest that the enzyme is not only carried by sperm in a soluble form within the acrosome but is also present on the sperm membranes [7]. In the present studies too centrifugation of whole sperm incubated with and without PI-PLC showed substantial amounts of NAGA in both supernatant and pellet fractions. While it is logical to assume that NAGA activity in the pellet is due to enzyme associated with sperm, the NAGA activity in the supernatant of samples with and without PI-PLC treatment is intriguing. Perhaps the NAGA activity in the supernatant is derived from a loosely adsorbed isoform of the enzyme released during the centrifugation. The enzyme firmly attached to sperm membrane is suggested to play a ligand-like role in binding the egg during the process of fertilization [18]. Alpha mannosidase is another sperm surface glycosidase that acts as a ligand for mannose residues found on egg vestments. Recently, this enzyme was reported to be GPI anchored in boar sperm [19]. Therefore we wanted to know how NAGA is associated with sperm membrane. The mode of anchorage of enzymes to the sperm surface often gives important clues to deciphering the possible mechanism by which it is acquired by sperm and hence this study is of interest.

The fact that NAGA was not released from sperm membrane preparation by repeated washing but readily solubilized with detergents like TX-100 and OG indicated that the enzyme was an integral not peripheral type of protein. It has been suggested before that GPI anchored proteins are better extracted by OG than triton X-100 at low temperatures. The reverse is true of other transmembranal polypeptides [20]. This is primarily due to the fact that GPI anchored proteins are associated with microdomains of the membranes called 'rafts' which are resistant to solubilization with non-ionic detergents. On the other hand, the resemblence of OG to components present in these rafts enables the solubilization of GPI proteins [21]. We used this criterion to check the pattern of solubilization of AP, a known GPI anchored protein and ATPase, which is not GPI anchored in microsomal membranes of goat kidney. The ratio of the specific activity of enzyme extracted by OG to that extracted by TX-100 was used as an index to judge if the NAGA attached to sperm membrane was GPI anchored. The pattern obtained was similar to that of ATPase and not of AP, indicating that sperm surface NAGA was not GPI anchored.

Using the same two microsomal enzymes as positive and negative controls we tried to see the pattern of enzymes released by PI-PLC. In general, GPI anchored membrane proteins are amphipathic molecules. The protein component together with its glycan backbone when cleaved and released from GPI anchors by PI-PLC becomes hydrophilic. Therefore, centrifugation after PI-PLC treatment would pellet the membrane and the released hydrophilic proteins would appear in the supernatant. On the other hand, proteins not attached via GPI anchors would pellet along with the membranes after PI-PLC treatment. In line with this notion PI-PLC was able to release AP but not ATPase from the microsomal fraction of goat kidney. NAGA from the sperm membrane preparation was also not released indicating that it was resistant to PI-PLC cleavage. Once again these experiments argue against a GPI anchorage for the association of NAGA on human sperm membranes. Just as in this study, NAGA of ascidian and drosophila sperm were also shown to be associated with sperm membranes but not through the GPI anchor [22,23].

Mammalian sperm require a large number of molecules including enzymes that could augment their chances of fertilizing the egg. Many of these are synthesized in testicular germ cells during the development of sperm. Unfortunately, due to its miniscule size it is unlikely that they would be able to cart all the molecules needed in the journey to meet with the egg. Besides, fully developed sperm lack endoplasmic reticulum and therefore has limited capacity for protein synthesis. The GPI-anchor by virtue of spanning only the outer leaflet of the lipid bilayer on sperm membranes facilitates lateral diffusion of molecules they hold. This not only economizes on the number of molecules needed but also enables better dispersion and interaction with other molecules on the sperm surface. The fact that NAGA is not GPI anchored means it cannot have the structural advantage of GPI-anchored molecules. Since NAGA is very important for fertilization related events, it follows that sperm will have to acquire this enzyme on a continuous basis from various sites along the male reproductive tract.

Dynamic changes leading to remodeling of lipid and protein components of the sperm surface are known to occur [12,24]. Culture studies have proved that NAGA is secreted by the epididymal epithelium [5]. Several routes of transport and transfer of proteins secreted by the male reproductive tract to the sperm are now known. A GPI anchor mediated cell-to-cell mechanism of protein transfer from epithelial cells of the male reproductive tract to the sperm surface has been suggested [25]. Another mechanism involving phospholipid transfer proteins (PL-TP) is also reported in literature [26]. Since our investigations did not support the view that NAGA is attached to the sperm membrane through the GPI anchor, we would like to examine other possibilities.

It has been suggested that high affinity sites that recognize fructose 6-phosphate derivatives (FPR-binding system) form part of a peculiar transport route that permits maturing spermatozoa to acquire hydrolytic enzymes secreted by the epididynal epithelium [27]. Whether NAGA is acquired by sperm in this fashion needs to be investigated. In rat epididymal fluid, membrane bound vesicles with NAGA activity have been isolated [28]. In human ejaculated semen too membrane bound vesicle characterized as prostosomes have been detected and shown to deliver enzymes like CD13/aminopeptidase to sperm by a fusion process [29,30]. Taken together, it seems more than likely that the enzyme is delivered from the epididymal epithelium to the sperm through membrane bound vesicles. However, more investigations would be needed to confirm this view.

While trying to decipher the exact mechanism of enzyme transfer, several issues also need to be addressed: We first need to know if NAGA is present on the membrane of testicular sperm. If so, whether the properties of the enzyme present on testicular, epididymal and ejaculated sperm remain the same. Further detailed analysis would be needed to clarify these points.

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