**N-Acetylglucosaminyltransferase I Activity in Bovine Ovarian Follicular Fluids from Dominant and Atretic Follicles**

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Abstract: The activity of α3-3′-mannoside-β1,2-N-acetylglucosaminyltransferase I (GnT I; EC 2.4.1.101), which catalyzes the first step in the conversion of oligomannose to complex or hybrid N-glycans of glycoproteins, was detected in bovine follicular fluid (bFF). The GnT I activity in bFF had a pH optimum of 5.8 and an absolute requirement for either Co2+, Mn2+, or Mg2+, the activity being stimulated by these cations in the above order. The apparent Km value for α3-3′-Ca mannopentaose of GnT I in bFF was 2.17 mM. The substrate specificity for the GnT I activity decreased in the following order: α3-3′-Ca mannopentaose > α1-3mannotriose > α1-3 mannobiose. The GnT I activity in bFF from large atretic follicles was significantly higher than in that from large dominant follicles. Moreover there was no significant difference between the GnT I activities in bFF from dominant follicles collected before and after surge of luteinizing hormone (LH surge). These data suggest that the GnT I activity in bFF may reflect functional changes in the microenvironment which lead to follicular atresia.

Key words: N-acetylglucosaminyltransferase I, dominant follicle, atretic follicle, follicular fluid, bovine

Development of a follicular wave in monovular species is a complex process in which a single follicle continues to grow and develop while the rest of the follicles cease growing and regress. The beginning of the difference in growth rates between the two largest follicles is termed "follicle deviation" and in cattle, that deviation occurs when the largest follicle reaches a mean diameter of 8.5 mm. The process of follicle selection is under systemic regulation by follicle-stimulating hormone (FSH) and LH as well as local regulation by factors that modulate the actions of gonadotropins. The selected follicle continues to grow as a dominant follicle, whereas the other follicles undergo atresia. Other factors such as follicle growth inhibitory factors may act independently of gonadotropins in follicle selection; however, their possible action has not yet been convincingly demonstrated.

In reproductive physiology, follicular atresia is a key phenomenon in follicle selection, which determines the species-specific number of newborn. The apoptosis that was first reported by Kerr et al. is a determinant in follicular atresia. It has been reported that sugar chain moieties of glycoconjugates on the cell surface are involved in apoptotic cell death. Since complex N-glycans are the most heterogeneous of the N-glycans of glycoproteins, these glycans may be involved in apoptotic cell death as well as in various biological phenomena as typified by congenital disorders of glycosylation type IIa. The conversion of oligomannose N-glycans to complex or hybrid N-glycans with antenna structures is catalyzed by N-acetylglucosaminyltransferases (GnT I to VI), α1,6-fucosyltransferase and α-mannosidases. The initial step in this process is catalyzed by GnT I, which suggests that high expression of GnT I activity may be involved in apoptotic cell death through the conversion of oligomannose N-glycans to complex or hybrid N-glycans of a key glycoprotein.

Glycosyltransferases catalyze the transfer of a sugar moiety from a sugar nucleotide as a donor to an acceptor. Those which are type II membrane proteins are generally localized in the Golgi apparatus and it has been reported that some of these enzymes are released through cleavage by an endogenous protease or proteases, and are then secreted from the cell. Indeed, many glycosyltransferases have been detected as soluble forms in extracellular fluids such as testicular and epididymal fluids, uterine and oviductal fluids as well as in serum, colostrum, amniotic fluid and milk. It has been documented, in addition, that proteolytic cleavage and secretion of glycosyltransferases into extracellular fluids are affected in various pathological conditions such as malignancy and inflammation as well as the progress of the estrous cycle. These findings suggest that the activities of soluble glycosyltransferases may have significant physiological roles, including apoptotic cell death, in some mammalian tissues.

In view of the above, we examined the soluble GnT I activity in the bovine follicular fluid (bFF) from large...
Materials. The following manno oligosaccharides were purchased from Funakoshi Co., Tokyo, Japan; α-1-3 mannoobiose [Man(α1-3)Man], α-1-6 mannoobiose [Man(α1-6)Man], α-1-3 α-1-6-mannotriosse [Man(α1-6)Man(α1-3)] Man and α-1-3 α-1-6-mannopenaose [Man(α1-3)Man(α1-6)Man(α1-3)Man]. Adenosine-5′-triphosphate (disodium salt) (ATP), D-N-acetylgalactosamine (O-GlcNAc) and uridine 5′-diphospho-N-acetylgalactosamine (disodium salt) (UDP-GlcNAc) were obtained from Sigma Co., St Louis, USA. UDP-[6-3H]-GlcNAc (16 Ci/ mmol) was from PerkinElmer, Inc., MA, USA. Bio-Rad AG1-X8 ion exchange resin (Cl− form, 200-400 mesh) was purchased from Bio Rad Laboratories, Hercules, USA.

Preparation of follicular fluid. The ovaries from Holstein cows were collected at a local slaughterhouse within 10–20 min after death and transported to the laboratory on ice. The stage of the estrous cycle was defined by microscopic observation of the ovaries. The bFF were aspirated from antral follicles using a disposable syringe with a 20-μgauge needle. Aspirated bFF was centrifuged at 1000 × g for 5 min to remove granulosa cells, then the weight of bFF was measured. The diameter (mm; D) of follicle was estimated using the following equation: 

\[ D = 14.077 \times W^{0.311}, \]

where W represents the weight (g) of bFF. This equation was obtained from a preliminary experiment in which the correlation between the weight of bFF and follicular diameter was examined (Murasawa et al., personal communication). The bFF from follicles whose diameter was over 8 mm were not pooled but were investigated individually; the bFF from small follicles, whose diameter was 2–6 mm, were pooled. All bFF samples were centrifuged to remove follicular debris and stored at −30°C prior to hormone or GnT I assay. All bFF samples were classified on the basis of their estradiol-17β (E2) / progesterone (P4) ratio. This was calculated from the E2 and P4 concentrations in bFF. The bFF from a dominant follicle was defined as having a E2/P4 ratio >1 while that from an atretic follicle had a E2/P4 ratio < 1.

Preparation of follicular fluids from collected follicles in vivo. Holstein cows were kept under the normal management program of the Center for Field Science of the university and fed with corn silage, hay and concentrate, with permanent free access to water. Experimental procedures complied with the Guide for Care and Use of Agriculture Animals of Obihiro University. To induce luteolysis all cows received 500 μg of a prostaglandin F2α (PGF2α) analogue (cloprostenol [estrumate]; Sumitomo Pharm. Co., Osaka, Japan) at the middle stage of the estrous cycle, and were assigned randomly as follows: Group 1) Follicular aspiration was performed at a luteal phase. A new follicular wave and ovulation of the preovulatory follicle was induced by a GnRH analogue (Fertirelin acetate 100 μg; [Conceral]; Takeda Pharm. Co., Osaka, Japan) injected 48 h after PGF2α. A dominant follicle was aspirated 7 days after GnRH injection, Group 2) Follicular aspiration was performed at follicular phase before LH surge. A dominant follicle was aspirated 42 h after an injection of PGF2α, Group 3) Follicular aspiration was performed at the follicular phase after LH surge. GnRH injected 48 h after PGF2α to induce endogenous LH surge. A dominant follicle was aspirated 24 h after GnRH injection. The day of follicular aspiration was designated as Day 0.

The dominant follicle of each cow was aspirated by transvaginal ultrasound-guided aspiration. For ultrasound guidance of the aspiration needle, an ultrasound scanner (SSD-5500, Aloka Co., Tokyo, Japan) equipped with a 7.5 MHz transvaginal convex transducer (UST-M15-21079, Aloka Co.) attached to an 18G stainless steel needle guide was used. When no dominant follicle could be identified under ultrasound, all follicles with diameters greater than 8 mm were aspirated. All bFF were brought to the laboratory on ice, and then centrifuged to remove follicular debris. The bFF samples were kept separately at −30°C until hormone or GnT I assay.

Estradiol-17β and progesterone determination. E2 and P4 concentrations in each of the follicular fluids were determined by double-antibody enzyme immunoassays (EIA) using 96-well ELISA plates (Corning Glass Works, Corning, NY), as described previously. Steroid assays were performed after diethyl ether extractions, follicular fluid being diluted 50 or 500-fold to permit measurement of E2 concentrations in EIA within the optimal range of the standard curve. The standard curve for P4 ranged from 0.05 to 50 ng/mL, and the effective dose (ED50) of the assay was 1.1 ng/mL. The intra- and interassay coefficients of variation (CVs) were 4.7 and 6.5%, respectively, and the recovery rate for P4, (1 ng) added to 1 mL samples was 95%. The standard curve for E2: ranged from 2 to 2000 pg/mL, and the ED50 of the assay was 72 pg/mL. The intra- and interassay CVs were 6.8 and 8.8%, respectively. The recovery rate of E2 was 75%.

Determination of GnT I activity of follicular fluid. GnT I activity was assayed by measuring the incorporation of [3H]-GlcNAc into the substrate mannopentaose, as described previously for GnT I in rat testicular and epididymal fluid.

The incubation mixture (100 μL) consisted of 50 mm sodium cacodylate buffer (pH 5.8), 20 mM MnCl2, 2 mm ATP, 200 mM D-GlcNAc, 1.5 mM UDP-GlcNAc containing UDP-[3H]-GlcNAc (10 kBq/ 100 μL incubation mixture), 6.7 mM α-1-3 α-1-6 mannopentaose and 10 μL of follicular fluid. Parallel incubations without an acceptor were performed as well. After incubation for 4 h at 37°C, the mixture was cooled in ice to stop the enzyme reaction and immediately passed through 4 mL of Bio-Rad AG1-X8 ion exchange resin (Cl− form, 200–400 mesh), contained in a Pasteur pipette, to remove residual UDP-[3H]-GlcNAc. Immediately after the elution, the incorporation of [3H]-GlcNAc was determined by liquid scintillation counting using a Packard Tri-Carbo scintillation counter.

The concentration of protein in bFF was determined by the Lowry method using bovine serum albumin as the standard.

Statistical analysis. Although the activities of GnT I were usually expressed with the amount of radio activity of the reaction products, the values for the GnT I activities were converted into relative ratios on the basis of the
The GnT I activity was characterized using bFF from dominant follicles.

**Optimum pH.**

Preliminary experiments with 50 mM sodium cacodylate buffer (pH 7.0) showed that bFF from both dominant and atretic follicles had significant GnT I activity, catalyzing the transfer of GlcNAc from UDP-GlcNAc to \( \alpha1-3\alpha1-6 \) mannopentaose. The GnT I activity of both fluids was optimal at pH 5.8. The data on Fig. 1 were obtained with the bFF from dominant follicles. The activities increased beyond pH 7.0 with sodium cacodylate buffer. The assay was performed with another buffer, i.e. Tris-HCl buffer to clarify if this increase is observed in the experiment using it, too. In subsequent experiments all measurements were done at the optimum pH. The GnT I activity increased linearly in proportion to the amount of these fluids present in the reaction mixture and to the reaction times (data not shown).

**Protein concentration in both dominant and atretic follicular fluids; specific activity.**

No significant differences in protein concentration between the bFF from dominant and atretic follicles or between bFF obtained before or after LH surge *in vivo* (4.3 +/- 0.1%) were detected. The highest specific GnT I activity was in the pooled bFF collected from atretic follicles whose diameter was 2 to 6 mm (21.1 +/- 1.7 nm products/h/mg protein of bFF), while the lowest was in bFF from a dominant follicle (12.2 +/- 2.0 nm products/h/mg protein of bFF). The characterization of the GnT I activity in bFF was performed using pooled bFF from dominant follicles.

**Km values and substrate specificities.**

The effects of changes in substrate concentration on GnT I activity are shown in Fig. 2. The apparent Km value for \( \alpha1-3\alpha1-6 \) mannopentaose, calculated form Lineweaver-Burk plots, was 2.17 mM.

Four different manno oligosaccharides, all tested at 6.7 mM, were examined as possible acceptors for the GnT I in bFF. Their effectiveness was in the order: \( \alpha1-3\alpha1-6 \) mannopentaose > \( \alpha1-3\alpha1-6 \) mannotriose > \( \alpha1-3 \) mannobiose. No activity was detected using \( \alpha1-6 \) mannobiose (Fig. 3).

**Effect of bivalent cations.**

The GnT I activity in bFF had an absolute requirement for either Mn\(^{2+}\) or another bivalent cation of which Co\(^{2+}\) was the most effective, followed by Mn\(^{2+}\) and Mg\(^{2+}\) in that order (Fig. 4). The optimum concentration of Mn\(^{2+}\) was 50 mM (Fig. 5). No GnT I activity could be demonstrated in either fluid when Zn\(^{2+}\), Cd\(^{2+}\) or Ca\(^{2+}\) was used instead of Mn\(^{2+}\).

**Comparison of GnT I activities in bFF from dominant and atretic follicles.**

The bFF from atretic follicles had higher GnT I activities than the bFF from dominant follicles, particularly in the case of the large follicles (Fig. 6). However, neither

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**RESULTS**

Fig. 1. Effect of pH on N-acetylglycosaminyltransferase I (GnT I) activity in bovine follicular fluid (bFF) from dominant follicles.

The GnT I activity was estimated as described in METHODS. The solid line and square (\(\text{□}^{-}\)) indicate the expression of GnT I activity in sodium cacodylate buffer, and the dotted line and circle (\(\text{●}^{-}\)) indicate the expression of the GnT I activity in Tris-HCl (50 mM, pH 7–9). Numbers in parenthesis indicate the repetitions of the experiment.

Inset shows the Lineweaver-Burk plot. Numbers in parenthesis indicate the repetitions of the experiment.

![Fig. 1](image1.png)

**Fig. 2.** Effect of substrate (\(\alpha1-3\alpha1-6\) mannopentaose) concentration on GnT I activity in bFF, measured at the optimum pH of 5.8 (see Fig. 1).

![Fig. 2](image2.png)
the bFF from dominant nor the bFF from atretic follicles showed significant differences of GnT I activity with respect to follicular size (data not shown). By the student t-test, no significant differences of the GnT I activities were observed among the following three follicular fluids: dominant follicles at middle luteal phase (diameter: 16.9 ± 1.5 mm), before LH surge (14.4 ± 2.8), and after LH surge (21.5 ± 2.2) (Fig. 7). But the differ-

DISCUSSION

This is the first report indicating that GnT I activities are present in mammalian follicular fluid. The properties of this enzyme activity, such as optimum pH and requirement for bivalent cations, were similar to those for the GnT I activities of rat liver, rabbit liver, and rat testicular fluid showing that this enzyme is GnT I, but were different from those for rat epididymal fluid as shown in Table 1. The enzyme has the substrate specificities for \( \alpha_1-3 \alpha_1-6 \) mannopentaose, \( \alpha_1-3 \alpha_1-6 \) mannotriose, and \( \alpha_1-3 \) mannobiose, but not for \( \alpha_1-6 \) mannobiose; this is typical substrate specificity for GnT I.

Glycosyltransferases are generally localized in the Golgi apparatus and require sugar nucleotides for the glycosylation. Since the presence of sugar nucleotides in follicular fluid has not as yet been demonstrated, it is still unclear whether carbohydrate modifications of glycoconjugates by soluble glycosyltransferases occur on the granulosa cell surface or on soluble peptides in the follicular fluid during follicular development. In addition one cannot exclude the possibility that glycosylation by soluble glycosyltransferases occurs in the extracellular fluid since sugar nucleotides as well as soluble glycosyltransferase activities have been detected in the milk or colostrum of some mammalian species.

Furthermore, Lazarowski et al. reported the release of UDP-glucose from human astrocytoma, bronchial epithelial cells, and rat glioma cells into the extracellular environment. These observations suggest that glycosylation may occur in the extracellular fluid. If UDP-GlcNAc were detected in bFF, this would suggest that carbohydrate modification of glycoconjugates on the granulosa cell surface or on the soluble peptides by this GnT I activity occurs in follicular fluid during follicular development.

The GnT I activity in bFF from an atretic follicle was
Table 1. GnT I activity of bovine follicular fluid; comparison with those of male genital tract fluids and liver of rat and rabbit.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Optimum pH</th>
<th>$K_v$ value (mM)</th>
<th>Effect of bivalent cation</th>
<th>Effective Mn²⁺ conc. (mM)</th>
<th>Specific activity *(^{\text{a}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine follicular fluid(^{1})</td>
<td>5.8</td>
<td>2.17</td>
<td>$\text{Co}^{2+} &gt; \text{Mn}^{2+} &gt; \text{Mg}^{2+} &gt; \text{Ca}^{2+}$</td>
<td>50</td>
<td>0.20</td>
</tr>
<tr>
<td>Rat testicular fluid</td>
<td>6.0</td>
<td>0.57</td>
<td>$\text{Co}^{2+} &gt; \text{Mn}^{2+} &gt; \text{Mg}^{2+} &gt; \text{Ca}^{2+}$</td>
<td>100</td>
<td>0.039</td>
</tr>
<tr>
<td>Rat cauda epididymal fluid</td>
<td>7.0</td>
<td>0.38</td>
<td>$\text{Mn}^{2+} &gt; \text{Ca}^{2+}$</td>
<td>50</td>
<td>0.044</td>
</tr>
<tr>
<td>Rat liver(^{3,5})</td>
<td>5.5</td>
<td>0.4</td>
<td>$\text{Co}^{2+} &gt; \text{Mn}^{2+} &gt; \text{Cd}^{2+} &gt; \text{Mg}^{2+} &gt; \text{Ca}^{2+} &gt; \text{Zn}^{2+}$</td>
<td>20–100</td>
<td>0.18*</td>
</tr>
<tr>
<td>Rabbit liver(^{5})</td>
<td>5.6</td>
<td>0.25</td>
<td>$\text{Mn}^{2+} &gt; \text{Co}^{2+} &gt; \text{Mg}^{2+} &gt; \text{Cd}^{2+} &gt; \text{Ca}^{2+}$</td>
<td>70</td>
<td>0.31</td>
</tr>
</tbody>
</table>

\(^{a}\) Collected from dominant follicles. \(^{1}\) a-1,3-%-l-6 mannopyranosae was used as a substrate for GnT I activities in both bovine follicular fluid and rat male genital tract fluids, while heptasaccharide peptide was used as a substrate for that in rat and rabbit liver. \(^{2}\) 'Zn⁺' and Cd⁺² had no effect on GnT I activity. \(^{3}\) At Mn⁺² concentration above 50 mM the cauda epididymal fluid became insoluble. \(*\) nmol of products amount/min/mg of protein. \(^{5}\) Enzyme activity of tissue homogenates.

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**ウシ優性卵胞と閉鎖卵胞の卵胞液中における**
**N-アセチルグルコサミルトランスフェラーゼＩ**

**活性の解析**

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ウシ優性卵胞と閉鎖卵胞の両卵胞液（bFF）中にβ2-
N-アセチルグルコサミルトランスフェラーゼＩ（GnT-I）
活性を検出した。本酵素活性は至適pH 5.8で示し、二
価亜硫酸イオン酸塩依存性（Co²⁺ Mn²⁺ Mg²⁺）と
基質特異性（α1-3α1-6マンノペンタオース＞α1-3α1-6
マンノトリオース＞α1-3マンノピオース）を有してい
た。α1-3α1-6マンノペンタオースに対するK₅は、2.17
mmであった。閉鎖卵胞のbFF中GnT-I活性は優性卵胞
のbFF中のそれより顕著に高かった。一方、ι-検定解析
により、優性卵胞のbFF中のGnT-I活性は LH サージの
前後で有意な変化を示さなかった。以上の結果から、顕
著に亢進した閉鎖卵胞のbFF中GnT-I活性は卵胞の閉
鎖化に関与している可能性が推察された。

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