Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 311-316

N-ACETYLGLUCOSAMINIDASE INHIBITOR ISOLATED FROM THE VITELLINE COAT OF ASCIDIAN EGGS IS A CANDIDATE SPERM RECEPTOR

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Received June 26, 1995

SUMMARY: An inhibitor against the egg β-D-*N*-acetylglucosaminidase (GlcNAcase) was purified from the vitelline coat of eggs of the ascidian, *Halocynthia roretzi* . The purified inhibitor gave a single band with an apparent molecular mass of 70 kDa on SDS-PAGE under non-reducing conditions. The GlcNAcase inhibitor potently inhibited the ascidian egg GlcNAcase activity, and also substantially inhibited the GlcNAcase activity in the ascidian sperm extract, although to lesser extent. In contrast, it showed little inhibitory effect on the activity of GlcNAcase from Jack beans. This inhibitor was found to block the binding of sperm to the vitelline coat of the eggs in a concentration-dependent manner. These results strongly suggest that the GlcNAcase inhibitor ioslated from the vitelline coat functions in the binding of sperm to the egg coat, as a possible sperm receptor.

Monospermic fertilization is indispensable for the normal embryonic development of most eggs. There are several mechanisms for prevention of polyspermy: blocking of the successive sperm-egg fusion by depolarization of the egg membrane potential triggered by the first spermatozoon, the hydrolysis or detachment of the sperm receptor on the vitelline coat by a trypsin-like protease (sperm receptor hydrolase) secreted from cortical granules of the eggs, and the formation and hardening of the fertilization membrane (1, 2).

In connection to the polyspermy blocking mechanisms in ascidians, Hoshi (3) first proposed a role of glycosidase (α-L-fucosidase in the case of *Ciona intestinalis*) located on the sperm cell surface, in the sperm binding to the vitelline coat, of which the sugar residue is a key substance of the sperm receptor (3). In the case of the ascidians, *Phallusia mammillata* (4, 5) and the genus *Ascidia* (6, 7), it has been reported that a sperm-associated β-D-*N*-acetylglucosaminidase (GlcNAcase) is responsible for the binding of sperm to the vitelline coat, in which cases β-D-*N*-acetylglucosamine (GlcNAc) is a candidate ligand for sperm binding. On the other hand, Lambert (6, 7) has proposed that a GlcNAcase, which is released from the

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Abbreviations used are: ASW, artificial seawater; BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole; FSW, filtered seawater; GlcNAc, *N*-acetylglucosamine; GlcNAcase, *N*-acetylglucosaminidase; 4MeUmb, 4-methylumbelliferyl; PAGE, polyacrylamide gel electrophresis; SDS, sodium dodecyl sulfate.

0006-291X/95 $12.00

eggs of *P. mammillata* and several *Ascidia* species into the surrounding seawater following fertilization, could bind to the GlcNAc moiety of the vitelline coat to block the binding of supernumerary sperm. Thus, the sugar-containing vitelline coat component, which gamete glycosidases can recognize, is a candidate sperm receptor and the egg glycosidase is thought to be responsible for the establishment of the polyspermy block in ascidian eggs.

In the previous paper (8), we purified GlcNAcase from the eggs of the ascidian, *Halocynthia roretzi*, and reported that this enyzme is capable of binding to the vitelline coat of the eggs. This finding led us to propose an idea that a vitelline coat component which is capable of interacting with the egg GlcNAcase is a candidate sperm receptor. In the course of our studies on vitelline coat components that can modurate the egg GlcNAcase activity, we found a GlcNAcase inhibitor in the extract of the vitelline coat of *H. rortezi* eggs. Here, we describe the purification procedure and some properties of this GlcNAcase inhibitor, and present evidence showing that this inhibitor is a candidate sperm receptor.

MATERIALS AND METHODS

Chemicals: Glycosidase substrates including 4-methylumbelliferyl β-D-*N*-acetylglucosaminide (4MeUmb-GlcNAc), 4-methylumbelliferone (4MeUmb), diisopropylfluorophosphate, and molecular mass standards were purchased from Sigma Chemical Company. DEAE-cellulose DE-32 and TSK-gel G3000 SW were obtained from Whatman Ltd. and Tosoh Ltd. (Tokyo, Japan), respectively. Ampholine, Superose 6, and Superose 12 are products of Pharmacia-LKB. Jack bean GlcNAcase and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Kapel and Nacalai Tesque (Kyoto, Japan), respectively. Leupeptin was a gift from Dr. Tetsuya Someno of Nippon Kayaku Co., Ltd. (Tokyo, Japan). Ascidian egg GlcNAcase was purified from eggs of *H. roretzi* as described previously (8).

Biologicals: Gametes from the ascidian, *H. roretzi*, type C, were collected as described previously (9). The eggs obtained from each individual were washed with filtered seawater (FSW) and stored at -40°C, while the spermatozoa were collected by centrifugation and stored at -40°C. Crude sperm extract was prepared as follows: the frozen sperm (0.25 ml) of *H. roretzi* were thawed, suspended in 50 mM Tris/HCl, pH 8.0, and sonicated at 50 W for 1 min. The homogenate was centrifugated at 10,000 x g for 30 min, and the resulting supernatant was used as a crude sperm extract.

Assays for GlcNAcase and Its Inhibitor: GlcNAcase activity was routinely measured at 25°C using 10 μM 4MeUmb-GlcNAc as a substrate as described previously (8). One unit of activity was defined as the amount of enzyme which hydrolyzes one μmol of 4MeUmb-substrate per min at 25°C.

GlcNAcase inhibitor activity was measured as follows: a reaction mixture (1.5 ml) containing 50 mM sodium acetate, pH 4.5, 1.2 munit of the ascidian egg GlcNAcase, and the egg inhibitor solution was preincubated at 25°C for 2 min. Then 4MeUmb-GlcNAc at a final concentration of 10 μM was added to the reaction mixture and the remaining activity was measured at 365 nm for excitation and at 445 nm for emission by tracing on a chart. One inhibitor unit was defined as the amount of inhibitor which inhibits one unit of the GlcNAcase activity under the above assay conditions.

Isolation of the Vitelline Coat: The frozen eggs (450 ml) of *H. roretzi* were thawed and homogenized in a 2-times volume of 50 mM Tris/HCl, pH 7.5, containing 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 μM benzamidine, 100 μM leupeptin, and 10 μM diisopropylfluorophosphate with a Teflon-glass homogenizer (480 rpm, 10 strokes) on ice. The homogenate was centrifuged at 800 x g for 30 min and then at 10,000 x g for 30 min, and the resulting precipitate was extensively washed at 4°C with 5 fold-diluted artificial seawater (ASW, 20 mM Tris/HCl, pH 8.0 containing 460 mM NaCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 10 mM KCl and 6 mM $NaHCO_3$) by pipetting and was filtered through a nylon mesh. The vitelline coat preparation thus obtained was apparently homogeneous as judged by light microscopy.

Purification of GlcNAcase Inhibitor from the Vitelline Coat: The vitelline coat (5 ml, wet volume) of *H. roretzi* eggs was extracted with 50 mM Tris/HCl, pH 8.0, containing 1% SDS at 37°C overnight. After the insoluble materials had been removed by centrifugation (3,000 x g, 10 min), the resulting supernatant was concentrated with an Amicon PM-10 membrane, and applied to a Superose 12 HR 30/10 column (3 x 10 cm) previously equilibrated with 25 mM Tris/HCl, pH 8.0, containing 200 mM glycine and 0.1% SDS (buffer A) using the Pharmacia FPLC system. The inhibitor activity was eluted around the void volume. Fractions having inhibitor activity were dialyzed against 8 M urea in order to thoroughly remove SDS, followed by dialysis against 50 mM Tris/HCl, pH 8.0, containing 0.1 % Lubrol (buffer B). The dialyzed sample was applied to a DEAE-cellulose (DE-32) column (3 x 15 cm) previously equilibrated with buffer B. After the column had been washed with buffer B, the inhibitor was eluted with a 0-1 M NaCl liner gradient in buffer B. The inhibitor activity was eluted at approximately 150 mM NaCl. Inhibitor fractions were concentrated using an Amicon PM-10 membrane, and dialyzed against buffer B, and were then applied to a Superose 6 HR 30/10 column (3 x 10 cm) previously equilibrated with buffer B using the Pharmacia FPLC system. The inhibitor activity emerged as a broad peak, which corresponded to the molecular mass of 500-1,000 kDa. Inhibitor fractions were dialyzed against 100 mM sodium phospate, pH 6.0, containing 0.1% SDS (buffer C), and was applied to a TSK G3000 SW column (0.5 x 60 cm) previously equilibrated with buffer C. The inhibitor activity emerged as a single peak which was superimposable with the protein peak (Fig. 1). The peak inhibitor fraction was referred to as the purified GlcNAcase inhibitor. Superose 12 and Superose 6 FPLCs were carried out at 0-4°C, while TSK G3000 SW gel filtration and DEAE-cellulose chromatography were performed at room temperature.

Protein concentration was determined by measuring the absorbance at 280 nm or by the method of Bradford (10) using bovine serum albumin (BSA) as a standard.

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli (11).

Preparation of Glycerol-Treated Eggs: Glycerol-treated eggs were prepared according to the method of Simard-Duquesne and Couillard (12) with modifications by Rosati and DeSantis (13). Unfertilized *H. roretzi* egg suspension was overlaid on a discontinuous density gradient (0%, 5%, 10%, 20%, 30% and 40%) of glycerol in FSW contained in a plastic centrifuge tube (50 ml), and the tube was kept cold (-20°C) until the unfertilized eggs sank to the bottom of the tube by gravity. These eggs at the bottom in FSW containing 40% glycerol were stored at -20°C for more than two days, and were then sequentially put into FSW containing 40%, 30%, 20%, 10%, and 0% of glycerol in this order to remove the glycerol.

Sperm Binding to Glycerol-Treated Eggs: Glycerol-treated eggs (20-40 eggs) were incubated for 10 min with *H. roretzi* sperm (about 10^6 cells) in the presence or absence of various concentrations of GlcNAcase inhibitor in SDS. The sperm-bound eggs were extensively washed with the ASW and fixed wth 4% glutaraldehyde in ASW for 30 min. The fixed specimens were stained with DAPI, a DNA-labeling reagent, for 30 min and the number of sperm which bound to one egg was counted on photographs taken by fluorescence microscopy. BSA was used as a control.

RESULTS AND DISCUSSION

Purification of GlcNAcase Inhibitor: The GlcNAcase inhibitor was purified to apparent homogeneity from the SDS-vitelline coat extract of eggs of *H. roretzi* by a procedure including gel filtration and ion-exchange chromatography (Table I). TSK-gel G3000SW gel filtration at the final purification step gave a single peak of inhibitor activity which is superimposable with the protein peak (Fig. 1). From 5 ml of packed volume of the vitelline coat, approximately 60 μg of the purified inhibitor was isolated with a yield of about 6%, estimated from the SDS-extract.

Properties of the GlcNAcase Inhibitor: The purified inhibitor preparation gave a single band on SDS-PAGE under non-reducing conditions (Fig. 1 inset). The molecular mass of the inhibitor was estimated to be 70 kDa. Under the reducing conditions, however, no detectable

Table I Purification of GlcNAcase Inhibitor from the Vitelline Coat of Ascidian Eggs

Step	Protein	Inhibitor activity	Specific inhibitor activity	Purification	Yield
	mg [a]	*units* [b]	*units/mg*	*-fold*	*%*
1. VC extract	9.7	2100	220	1.0 [c]	100
2. Superose 12	2.0	180	90	0.4	8.6
3. DEAE-cellulose	0.41	190	450	2.1	8.8
4. Superose 6	0.11	120	1100	5.1	5.7
5. TSK G3000 SW	0.06	120	2000	9.3	5.7

[a] Protein concentration in step 1-4 was determined by the method of Bradford (10) with BSA as a standard, while that in step 5 was determined by measuring the absorbance at 280 nm and assuming that $A^{1\%}_{280} = 10.0$.

[b] One inhibitor unit was defined as the amount of inhibitor which inhibits one unit of ascidian egg GlcNAcase.

[c] Defined as 1.0.

band with a molecular mass of more than 10 kDa was observed, suggesting that this inhibitor is composed of several low molecular mass (less than 10 kDa) polypeptides which are thought to be cross-linked via disulfide bond.

The effects of the purified inhibitor on the activities of GlcNAcases from ascidian eggs, ascidian sperm, and Jack beans are compared and depicted in Fig. 2. The GlcNAcase inhibitor

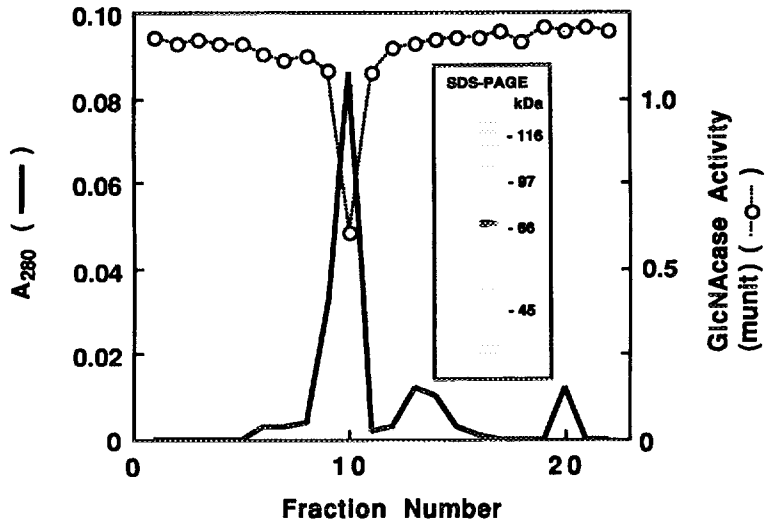
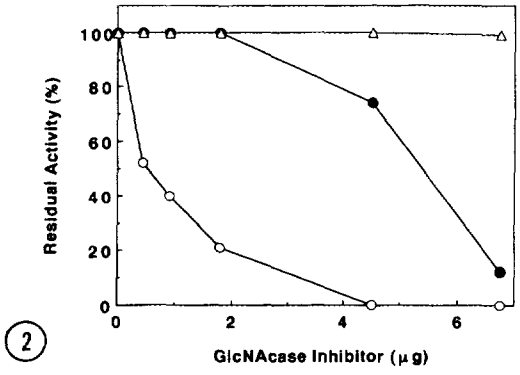


Fig. 1. TSK G3000 SW gel filtration of the ascidian egg GlcNAcase inhibitor. The GlcNAcase inhibitor preparation obtained from Superose 6 column was subjected to gel filtration on TSKgel G3000 SW column (0.5 x 60 cm) previously equilibrated with buffer C. The inhibitor activity was eluted in a single peak. Fractions of 1ml were collected at a flow rate of 0.5 ml/min. —, A_{280}; - - o - -, GlcNAcase activity (unit/ml). *Inset*, The purified GlcNAcase inhibitor was subjected to SDS-PAGE under non-reducing conditions and stained by silver staining.

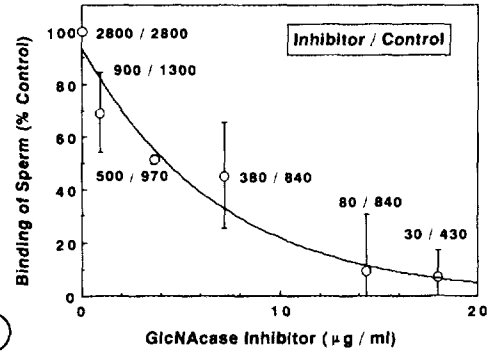
inhibited the egg GlcNAcase most potently among the three GlcNAcases so far tested, and substantially inhibited the GlcNAcase of ascidian sperm origin. On the other hand, it showed little inhibition against the Jack bean GlcNAcase.

To ascertain whether the GlcNAcase inhibitor can bind to the egg GlcNAcase under physiological conditions (i. e., in the ASW), a mixture of the enzyme and its inhibitor was subjected to gel filtration on a Sepharose 6B column equilibrated with ASW. It was found that the egg GlcNAcase with a molecular mass of 520 kDa (8) emerged at approximately 320 kDa (data not shown). This result suggests that the enzyme interacts with the inhibitor under physiological conditions. Furthermore, it is thought that the inhibitor may disrupt the oligomeric nature (8) of the enzyme to give a smaller molecular form.

Effect of the GlcNAcase Inhibitor on the Binding of Asdidian Sperm to the Vitelline Coat: In order to elucidate whether the isolated GlcNAcase inhibitor is a potential sperm receptor located on the vitelline coat, we examined the effect of the GlcNAcase inhibitor on the binding of the ascidian sperm to the vitelline coat of ascidian eggs (Fig. 3). The binding of sperm to the vitelline coat of the glycerinated eggs was diminished by the GlcNAcase inhibitor in a dose-dependent manner, compared with the effect of the same amount of BSA, used as a control. These results, together with the fact that this inhibitor inhibited the sperm GlcNAcase activity, strongly suggest that the GlcNAcase inhibitor isolated from the vitelline coat is a candidate for sperm receptor. The fact that lectins specific for GlcNAc effectively blocked fertilization (8) is consistent with the above assumption.



(2)



(3)

Fig. 2. Inhibition of various GlcNAcases by the ascidian egg GlcNAcase inhibitor. Each enzyme (1.2 units) was preincubated with the GlcNAcase inhibitor and the remaining activity toward 4MeUmb-GlcNAc was measured. —○—, GlcNAcase from eggs of *H. roretzi* ;—●— GlcNAcase from sperm of *H. roretzi* ;—△— GlcNAcase from Jack bean.

Fig. 3. Inhibitory ability of the GlcNAcase inhibitor against the binding of sperm to the vitelline coat of glycerinated eggs. Glycerinated eggs of *H. roretzi* were incubated with *H. roretzi* sperm in the presence or absence of GlcNAcase inhibitor in SDS. BSA was used as a control. Note that the final concentration of SDS also varried with that of the inhibitor on the basis that the inhibitor at 10 μg/ml contained about 0.01 % SDS. The ordinate indicates the ratio of the number of sperm bound to the vitelline coat of glycerinated eggs in the presence of the GlcNAcase inhibitor to that in the presence of BSA at the same concentration dissolved in the same SDS concentration.

In mice, a zona glycoprotein, ZP3 (83 kDa), is thought to be a sperm receptor located on the egg coat (zona pellucida) because acrosome-intact sperm can bind to the ZP3 to undergo the acrosome reaction (14, 15). The oligosaccharide portion of ZP3 is responsible for the sperm receptor activity (16). It has been reported that the sperm surface-bound enzyme (β-1,4-galactosyl transferase in this case) and the ZP3 are complementary adhesion molecules that mediate sperm-egg binding in the mouse (17). Basic mechanisms of sperm egg binding are thought to be common in most animals including ascidians.

Acknowledgments: We are indebted to Dr. Takaharu Numakunai, Asamushi Marine Biologiclal Station, Tohoku University, for his cooperation in collecting the animals.

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