MAMMALIAN SPERM ACROSOMAL NEURAMINIDASES

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Received March 20, 1970

Summary: A neuraminidase and a neuraminidase-like factor (NLF) were demonstrated for the first time in acrosomes of rabbit, bull, hamster, ram and human spermatozoa. The neuraminidase is similar to the known mammalian neuraminidases, but the NLF is unique in that it renders bound sialic acid reactive in Warren's colorimetric assay but does not release it from the macromolecular complex. Partially purified decapacitation factor preparations that inhibit fertilization also inhibit NLF activity. The true neuraminidase activity appears to arise from NLF during purification. Cowpers gland mucin of the boar was used as substrate, and the effect of neuraminidase and NLF on this substrate was not duplicated by trypsin, α-chymotrypsin, pronase, α-amylase, lysozyme, hyaluronidase or detergents.

We have undertaken study of the hydrolytic enzymes of spermatozoa that may be involved in penetration and fertilization of the ovum. We have found neuraminidase and a neuraminidase-like factor (NLF) present in extracts of sperm acrosomes that may be involved in fertilization. While this manuscript was under revision, Brown, Srivastava and Hartree (1) reported a factor in the rabbit uterus and other animal tissues that causes sialyl residues of mucoproteins to react as free sialic acid in the Warren assay (2). This factor (UF) and NLF have some similarities and some differences in their properties.

MATERIALS AND METHODS

Materials: Neuraminidase from Clostridium perfringens, N-acetylneuraminic acid (NANA), N-acetyl neuramin lactose (bovine colostrum), Mucin (porcine stomach crude type II), and Mucin Type I (BSM = bovine submaxillary glands) were purchased from Sigma Chemicals. Muramidase (2 x crystallized lysozyme), α-amylase, pancreatic trypsin (2 x crystallized), α-chymotrypsin (3 x crystallized) and Mucin MU 8AA were the products of Worthington Biochemical Corporation. Glycoprotein

Fraction VI (bovine) and Sialic Acid Concentrate (from beef submaxillary gland) were obtained from Nutritional Biochemical Corporation. Pronase grade B and Neuramin lactose (beef colostrum) were purchased from Calbiochem. Hyamine 2389 was obtained from the British Drug House and Triton X-100 from Mann Research Laboratories.

Analyses: Protein was estimated by the method of Lowry *et al.* (3) using bovine serum albumin as standard. Sialic acid was estimated by the method of Warren (2) and NAN-aldolase by method of Brunetti *et al.* (4).

Preparation of NLF: Acrosomal extracts were prepared from batches of pooled semen from 40 rabbits ejaculated by artificial vagina. The method of Hartree and Srivastava (5) was essentially followed except the detergent treatment step also included 0.1% (v/v) of Triton X-100 besides Hyamine 2389. The supernatant solution containing the acrosomal extract was precipitated either with 4 volumes of ethanol pre-cooled to -15 C or with 80% saturated $(NH_4)_2SO_4$. The precipitates were dialyzed at 4 C against 100 volumes of glass distilled water containing 50 µg penicillin G and 50 µg streptomycin sulfate per ml. These levels of antibiotics were maintained in all experiments. The non-diffusible residue was stored below 0 C until assayed.

Substrate - Cowper's Glands Mucin (CGM) of the Boar: The colorless viscous secretion was squeezed from the glands. A weighed amount was suspended in buffer at the desired pH and stirred at room temperature for several hours until the secretion went into solution. The solution was dialyzed against 10 volumes of buffer for three days at 4 C changing the buffer every day. The non-diffusible residue was used as the substrate.

Estimation of Enzyme Activity: The reaction was carried out in a total volume of 1 ml. The reaction mixture contained buffer, enzyme and substrate. Suitable reagent blanks were maintained. After incubation at 37 C, 0.2 ml of the mixture was assayed for free sialic acid. One unit is defined as that amount of enzyme which will liberate 1 µM sialic acid per min from the CGM at 37 C and pH 6.0. Specific activity is expressed as units/mg protein.

RESULTS AND DISCUSSION

Differences Between the Known Neuraminidases and NLF in their Action on CGM: In a dialysis bag 125 µg of bacterial neuraminidase from *Cl. perfringens* (specific activity = 0.03 units/mg on sialyl lactose) was incubated with 20 mg CGM and 2.5 ml of 0.2 M Tris buffers, pH 6.0, at 37 C while being dialyzed against 100 ml distilled water for 8 hours. Similarly, 60 µg of the acrosomal protein (specific activity = 0.03 units/mg on CGM) was incubated with 8 mg CGM. The diffusates from the two were evaporated *in vacuo* to a small volume. Estimations of sialic acid were made in the diffusates and the non-diffusible residues in the bag. Bacterial neuraminidase liberated 98% of the total sialic acid from CGM out of which 92% was detected in the diffusate. In contrast, sperm neuraminidase made 25% of the bound sialic acid in the dialysis bag reactive in the Warren test, but only traces of sialic acid were found in the diffusate. However, when 0.11 µM of sialic acid was incubated in the bag with substrate and enzyme, the sialic acid was recovered quantitatively in the diffusate. Therefore, the non-diffusibili could not be due to the formation of non-diffusible enzyme-product complex. It is most likely that NLF preferentially attacks the substrates with 2 → 6 linkages between sialic acid and sugar since it hydrolyzes sialyl lactose which has a 2 → 3 linkage very slowly. Since bacterial neuraminidase releases 98% of the bound sialic acid from CGM and NLF makes about 25% reactive in the color test, it would appear that 73% of the sialic acid in the CGM is resistant to NLF. It is proposed that NLF reacts with the sialic acid moiety of the substrate in such a way that the 2-ketosidic linkage is modified to form the chromogen under the conditions of Warren's assay for free sialic acid.

Inhibition of NLF by Rabbit Decapacitation Factor Preparations (DF): Decapacitation factor (6) is a substance found in seminal plasma which functionally reverses the capacitation process and thus prevents sperm penetration into the ovum. Pooled acrosomal extracts with a specific activity of 0.08 units/mg were tested for inhibition by partially purified DF prepared from rabbit and bull seminal plasma (column fraction 2) (7). One hundred µg of NLF was first incu-

bated with 150 µg DF at 37 C for 5 min, then 8 mg CGM was added and the mixture incubated further for 10 min. At this level, the DF preparation completely inhibited NLF. Decreasing the levels of DF gave a graded inhibitory response. Bacterial neuraminidase, on the other hand, was only slightly inhibited by DF. A mixture containing NLF, DF and the substrate was dialyzed at 4 C for 6 hr, the DF passing into the diffusate. On addition of more substrate to the non-diffusible residue containing NLF, the same level of NLF activity was obtained as in the control without DF. Therefore, inhibition of NLF was not due to the modification of the substrate by DF. Furthermore, on the addition of the diffusate which contained the DF, the activity was again inhibited. DF also inhibited NLF prepared from bull and human sperm. It should be pointed out that the DF preparation tested was still not pure and contains components other than DF which might inhibit NLF. Therefore, until pure DF is available, the possibility that the inhibition of NLF may well be due to contaminating components in the preparations cannot be excluded.

Estimation of NLF in Acrosomal Extracts: A total of 20 acrosomal extractions, each prepared from pooled sperm of 40 rabbits, were assayed for their action on CGM. Initially, a comparative study was made on the efficacy of the method of extraction. The average specific activity of 10 extracts prepared by alcohol precipitation and dialysis was 0.045 units/mg (range, 0.01 to 0.1) and that of 10 extracts obtained by $(NH_4)_2SO_4$ precipitation and dialysis was 0.092 units/mg (range, 0.03 to 0.3). NLF activity was also present in the acrosomal extracts of bull, ram, hamster and human spermatozoa. CGM (8 mg) was incubated separately with 1.2 mg muramidase, 3.5 mg α-amylase, 1 mg trypsin, 1 mg α-chymotrypsin 1 mg pronase, 1000 units of hyaluronidase, 10 µg Hyamine and 10 µg Triton X-100. None of these released sialic acid from CGM in the Warren test.

Effect of Detergents on the Extraction of the Enzyme: A pooled collection of sperm from 40 rabbits was split in two parts after washing. One part was treated with Hyamine and the other with Triton X-100. The acrosomal extract prepared by the Hyamine treatment alone, 0.9 mg protein from 134×10^6 sperm, had

very little NLF activity (specific activity 0.005), while that prepared by Triton treatment alone, 1.35 mg protein from $99x10^6$ sperm, had no activity. However, when the Hyamine treated sample was homogenized with Triton it showed high activity (specific activity 0.3). On ultracentrifugation at 160,000g, out of 3.25 mg protein, 2.75 was present in the supernatant and 0.5 mg in the sediment. Of the total NLF activity 90% was in the supernatant and 10% in the sediment. These results indicate that Hyamine is required to detach the acrosomes from spermatozoa and then the enzyme is solubilized from the acrosome by Triton. In contrast, after ultracentrifugation of UF at 100,000g the activity is entirely associated with the sediment (1).

Effects of pH, Enzyme Concentration and Incubation Time: These results

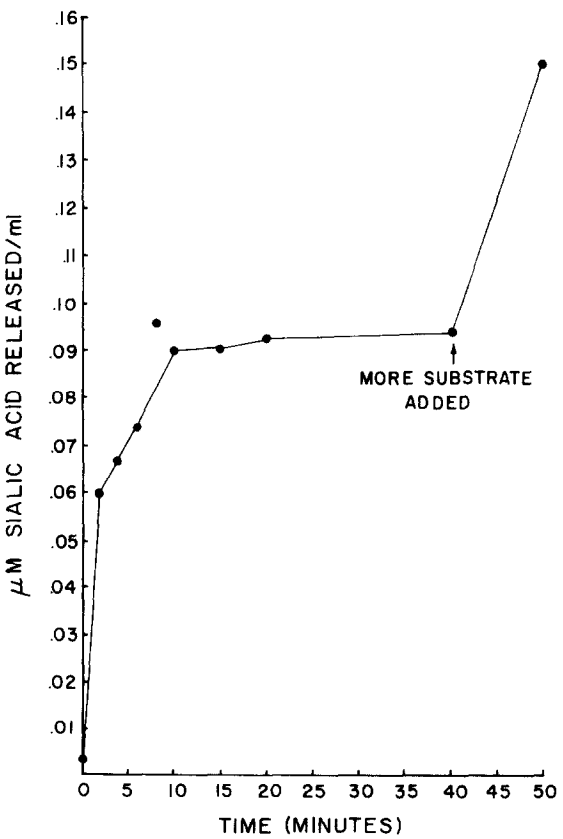

Fig. 1. The NLF activity of the acrosomal extract on CGM. The reaction mixture containing 30 μg acrosomal protein in 0.5 ml of 0.2 M Tris-maleic acid buffer pH 6.0, 4 mg substrate in 0.2 ml buffer and 0.3 ml buffer was incubated at 37 C.

pertain to the crude enzyme preparations obtained by $(NH_4)_2SO_4$ precipitation and dialysis. The crude acrosomal extract has maximum NLF activity at pH 6. The rate of reaction is almost linear during short periods of incubation and then levels off on further incubation. Addition of more substrate at 40 min results in further color in the Warren test (Fig. 1). Increasing the concentration of the substrate beyond the optimum level results in high viscosity and inhibition of enzyme activity. In many experiments, the sialic acid reacting after enzyme action appears to drop off on further incubation, possibly because of contamination by NAN-aldolase in crude extracts. However, the estimation for NAN-aldolase by the method of Brunette, Jourdian and Roseman (4) did not show enough aldolase present to justify the differences in the levels of sialic acid.

Substrate Specificity: The activity of NLF was tested on several known forms of sialic acid (Table 1). Rather low rates of hydrolysis of sialyl lactose and BSM indicates the inefficiency of attack on the 2 → 3 linkages present in

Table 1

Bound Sialic Acid Rendered Reactive by NLF

Substrates	Total µg Sialic Acid in the Substrate	% of Total Sialic Acid Reactive after NLF
CGM	30	31
Mucin MU8AA	24	4
Mucin (BSM)	12	6
Mucin (Porcine Stomach)	12	2.5
Sialyl lactose (Bovine Colostrum)	27	5
Glycoprotein fraction VI	39	1
Sialic Acid Concentrate prepared from BSM and dialyzed	34	10

The reaction mixtures consisted of 0.1 ml NLF (75 µg protein) and 0.1 ml substrate. After 10 min incubation at 37 C, sialic acid was estimated by Warren's method on the digests without dialysis. Total sialic acid in the substrate was estimated after acid hydrolysis.

these two substrates (8). Unlike bacterial neuraminidase which almost completely releases sialic acid from CGM and other substrates, mammalian neuraminidases in general exhibit much lower rates of hydrolysis (20 to 30%) of these substrates even after prolonged incubation (9). Calf brain neuraminidase does not hydrolyze sialyl lactose and "Sialic Acid Concentrate" (10). Rat liver neuraminidase released only 2% sialic acid from sialyl lactose (11). The specific activity of NLF prepared by $(NH_4)_2SO_4$ precipitation is several times greater than preparations from chick embryo (12) and blood plasma neuraminidase (13) and is comparable to UF (1), but is below that of viral and bacterial neuraminidase (14, 15).

The lack of effect of NLF on CGM from which all the sialic acid is released and removed by dialysis after treatment with bacterial neuraminidase indicates the NLF reacts with bound sialic acid and not with any other component of the macromolecule.*

Heat Stability: Thermostability of NLF preparations was variable. In 15 samples heated to 100 C for 10 min at pH 6, the activity was completely abolished in 8, partly in 3, and 4 appeared to be thermostable. On the other hand, UF has not inactivated even after heating to 130 C (1).

Evidence for the Enzymatic Nature of NLF: Chromatography of the crude acrosomal extracts of rabbit or bull spermatozoa on DEAE cellulose at 5 C results in five major protein peaks. With both sources, the first peak contains neuraminidase activity similar to the known neuraminidases, i.e., it is capable of releasing bound sialic acid from CGM and the free sialic acid is dialyzable. Finally, this neuraminidase activity can be abolished completely by heating at 100 C for 5 min. The remaining protein peaks contain NLF activity. However, this activity is masked and requires removal of salts before being apparent. Major difference in the first protein peaks of bull and rabbit acrosomal extract is that in bull sperm extracts there is no contamination by NLF; in rabbit both NLF and the

*This experiment was suggested by Dr. Saul Roseman, Johns Hopkins University, Baltimore, Maryland.

neuraminidase are present in about equal amounts. Both NLF and the neuraminidase of bull sperm have high molecular weights as these are not retarded on Sephadex G-75 columns. Recent evidence indicate that NLF and neuraminidase are interconvertible. The possibility of the bacterial generation of the neuraminidase activity in the acrosomal extracts by contamination is ruled out as suitable precautions were taken at all steps.

ACKNOWLEDGMENTS

The authors are indebted to Mr. R. T. Robertson and Dr. V. K. Bhalla for purified DF.

This research was supported by National Institutes of Health Research Grant HD GM 02799 and Training Grant 5 TO1 HD-00140 from the National Institute of Child Health and Human Development and Career Development Award 2 K3 GM4831 from National Institute of General Medical Sciences.

This work was also supported by a Ford Foundation Grant.

We thank Leroy Patrick and Ora Lee Johnson for their assistance in care of the animals and in surgery.

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