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A Modified and Improved Assay for Sperm Amidase Activity

NICHOLAS L. CROSS

From the Division of Reproductive Biology and Medicine, Department of Obstetrics and Gynecology, University of California, Davis, California

An assay for sperm amidase activity has been modified so that many samples can be measured rapidly, making it more suitable for use in a clinical setting. The modified assay gave the same results ($P > 0.05$) as its lengthier parent assay (Kennedy et al, 1989). The measured enzyme activity was proportional to the number of sperm assayed over the range tested (3.5×10^5 to 2.1×10^6 sperm). The within-assay coefficient of variation was 7.8 ± 1.8% (average ± SE, n = 12), and the between-assay coefficient of variation was 7.2 ± 1.2% (n = 3). The sensitivity was 7.6 μIU/well.

Key words: amidase, acrosin, acrosome, protease

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Activity of the sperm acrosomal protease, acrosin, may be correlated with male fertility (Goodpasture et al, 1982; Mohsenian et al, 1982; Kennedy et al, 1989), and may give an independent measure of sperm function (Goodpasture et al, 1982; Koukoulis et al, 1989). Conventional acrosin assays are lengthy, require many sperm, and consist of many steps. Measurement of the proteolytic activity of acrosin is cumbersome (Schleuning and Fritz, 1976), so assays have been devised to exploit its esterolytic or amidolytic activity (Polakoski et al, 1977; Schleuning and Fritz, 1976). Recently, a simplified procedure was reported (Kennedy et al, 1989). It employs a single solution containing a detergent to expose acrosomal material, a buffer at pH 8.0 to activate proacrosin to acrosin, and N α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as a chromogenic substrate for acrosin amidase activity. Although the assay measures benzamidine-sensitive amidase activity rather than extractable acrosin activity, Kennedy et al (1989) determined that those two assays gave the same results, and concluded that all available evidence supported the notion that their amidase assay was entirely or primarily due to acrosin. The purpose of the present work was to further shorten their assay by eliminating two unnecessary steps and by adapting it for use in 96-well microplates. These changes make it suitable for assaying many samples simultaneously.

Materials and Methods

To separate sperm from seminal plasma, aliquots of semen were layered over 0.5 ml of Ficoll solution (11%, w/v, Ficoll, 120 mmol/L NaCl, 25 mmol/L Hepes, pH 7.4) in a 5 ml conical polypropylene centrifuge tube, and cen-

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Correspondence to: Nicholas L. Cross, Division of Reproductive Biology and Medicine, Department of Obstetrics and Gynecology, University of California, Davis, CA 95616.
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trifuged at 1000g for 30 minutes at room temperature (Kennedy et al, 1989). After centrifugation, a pet nail clipper was used to cut through the tube below the interface between the seminal fluid and the Ficoll solution, so that the seminal plasma was completely removed. The pelleted sperm were then suspended in about 100 μl of Ficoll solution. Duplicate aliquots of this suspension were diluted 100-fold and the sperm concentrations determined with a hemacytometer. The sperm suspension was then adjusted to 7.0×10^7/ml in Ficoll solution.

Except where otherwise noted, the following were placed in the wells of a 96-well microplate (Linbro Division, Flow Laboratories, Inc; Hamden, CT). "Blank" wells contained 21 μl of Ficoll solution, 210 μl of substrate–detergent solution (2.3 mmol/L BAPNA, 10%, v/v, dimethylsulfoxide, 0.009% w/v Triton X-100, 49.5 mmol/L Hepes, and 49.5 mmol/L NaCl, pH 8.0; see Kennedy et al [1989] for a detailed description of how to prepare this solution). "Control" wells contained 21 μl of sperm suspension, 21 μl of 500 mmol/L benzamidine, and 210 μl of substrate–detergent solution. "Test" wells contained 21 μl of sperm suspension and 210 μl of substrate–detergent solution. The plates were incubated at room temperature (22 to 23°C). After 3 hours, the absorbances were determined using a Dynatech MR600 Microplate Reader (Alexandria, VA), set in the dual wavelength mode with λ_{test} = 410 nm and $\lambda_{reference}$ = 490 nm, and the blank set to zero.

In this protocol, the optical absorbance of the reaction product (p-nitroaniline, PNA) is determined without removing the sperm from the reaction mixture. The sperm make the solution turbid, increasing the absorbance. The absorbance is corrected for the contribution due to sperm by measuring in the dual wavelength mode, and by subtracting the absorbance of a control solution that contains an equal number of sperm. The accuracy of the correction procedure was tested in two ways. First, the absorbances of reaction mixtures were determined before and after removing the sperm by centrifugation. These assays were done in tubes containing 35 μl of sperm suspension and proportionally increased volumes of the other reagents. At the end of 3 hours of incubation, the reaction was stopped by adding 35 μl of 500 mmol/L benzamidine to the test and blank tubes. Aliquots (252 μl) were placed in microplate wells and their absorbances determined. The aliquots were then returned to the tubes and the sperm sedimented by centrifugation at 1000g for 30 minutes. A 252 μl aliquot of the sperm-free supernatant was removed and the absorbance was determined again. For each measurement, the absorbances of the control replicates were averaged and subtracted from the absorbances of the matching test aliquots to obtain the corrected absorbances.

In the second approach, the number of sperm was varied while the PNA concentration was held constant. Solutions of PNA were prepared, and the ability of the correction procedure to remove the absorbance due to added sperm was tested. The composition of the PNA solutions was identical to that of the substrate-detergent solution except that BAPNA was replaced with either 22, 68, or 153 μmol/L PNA. The contents of the microplate wells were as follows: "blank" wells contained 21 μl of Ficoll solution and 210 μl of buffer solution (substrate-detergent solution without BAPNA or PNA); "reference" wells contained 21 μl of Ficoll solution and 210 μl of PNA solution; "test" wells contained 21 μl of sperm suspension (containing 1.0×10^5 to 2.5×10^6 sperm) and 210 μl of PNA solution; "control" wells contained 210 μl of buffer solution and 21 μl of either Ficoll solution (in the control wells that were paired with reference wells) or sperm suspension (in the control wells that were paired with test wells). Two or three replicate wells were prepared. The absorbances of the wells were determined in the dual wavelength mode, with the blank set to zero. The absorbances of the replicate control wells were averaged and this value was subtracted from the absorbance of each matching reference or test well to give the corrected absorbance. If the correction procedure is appropriate, then the corrected absorbances of reference and test wells should be the same for a given concentration of PNA.

Calculation of Enzyme Activity

A millimolar extinction coefficient of 9.9 $mmol^{-1}\ L^{-1}\ cm^{-1}$ was used to convert the optical absorbance to moles of PNA produced by the hydrolysis of BAPNA. A factor was also required to correct for the short optical path length of the microplate wells. Six solutions containing PNA in concentrations ranging from 20 to 100 μmol/L were prepared, and the absorbance of each solution was measured using both a spectrophotometer (at 410 nm, using 1.0 cm path-length cuvettes) and a microplate reader as described above (252 μl aliquots). The factor was determined by dividing the absorbance measured with the microplate reader into the absorbance measured with the spectrophotometer. The result was 1.80 ± 0.008 (mean ± SE, n = 6). This factor should be determined independently for other microplates or plate readers.

One IU of acrosin activity is defined as the amount of enzyme that hydrolyzes 1 μmol BAPNA/minute at 23°C. Given that each well contains 1.5×10^6 sperm, the assay is 180 minutes long, and the volume is 0.252 ml, the μIU per million sperm is

$$\frac{[\text{mean OD of test} - \text{mean OD of control}] \times 1.8 \times 0.252 \times 10^6}{[9.9 \times 180 \times 1.5]}$$
$$= 170 \times [\text{mean OD of test} - \text{mean OD of control}].$$

This simplified assay was compared with its parent assay (Kennedy et al, 1989), in which the control and test assay tubes contained 100 μl of sperm suspension and proportionally increased volumes of the other reagents. At the end of 3 hours, 100 μl of benzamidine solution was added to blank and test samples. Tubes were centrifuged at 1000g for 30 minutes to allow sedimentation of the sperm, after which the absorbance of the supernatants was determined at 410 nm using a spectrophotometer.

Statistics

Comparisons of the old and new protocols, the absor-

bances in the presence or absence of benzamidine, and tests of the correction procedure were analyzed by paired sample t-tests. Regression lines were calculated by the method of least squares. To determine the between-assay coefficient of variation, an ejaculate was divided into four aliquots, and the assay performed on each aliquot using independently prepared reagents. This was done on three occasions, each time with a different ejaculate and different solutions. The within-assay coefficient of variation was calculated from the results of 12 assays (each of a different ejaculate) that employed four to eight replicates per assay.

Results

In the microplate assay, the absorbance of the reaction product was determined in the presence of sperm. The sperm contribute to the absorbance, so the measured absorbance was corrected. Two approaches measured whether the correction accurately removed the sperm-associated absorbance. First, aliquots of reaction mixtures were analyzed before and after removing the sperm by centrifugation. A very small but significant difference was found ($P < 0.05$). The absorbance corrected for the presence of sperm was 3.1% ± 1.0% (mean ± SE, n = 18) greater than that determined in the absence of sperm, over the range of 18 to 76 μIU/10^6 sperm (all of these reaction mixtures contained 1.5×10^6 sperm/252 μl). Second, the absorbances of three different concentrations of PNA were determined in the absence of sperm, and in the presence of increasing numbers of sperm. The results (Fig 1) again indicated that there was little difference between the absorbance of the reaction product in the absence of sperm and the value corrected for the presence of sperm. The percentage error was small (Fig 2), and significantly correlated with the number of sperm ($r = 0.59$, $P < 0.01$). At the recommended 1.5×10^6 sperm/well, the error was about 3%.

The simplified microplate acrosin assay was compared with its lengthier parent assay, and it gave the same results ($P > 0.05$, n = 11; Table 1). The measured enzyme activity was proportional to the number of sperm/well (Fig 3) over the range of sperm numbers tested (3.5×10^6 to 2.1×10^6). The between-assay coefficient of variation for the microplate assay was 7.2 ± 1.2% (average ± SE; n = 3), and the within-assay coefficient of variation was 7.8 ± 1.8% (mean ± SE, n = 12, with four to eight replicates per assay). It was not necessary to add benzamidine to the blank and test wells because benzamidine did not contribute to the absorbance of the solution ($P > 0.05$) and because the sample reading is completed within 1 minute. The sensitivity, defined as the amount of activity giving an absorbance equal to twice the standard deviation of the control well absorbance, was 7.6 μIU.

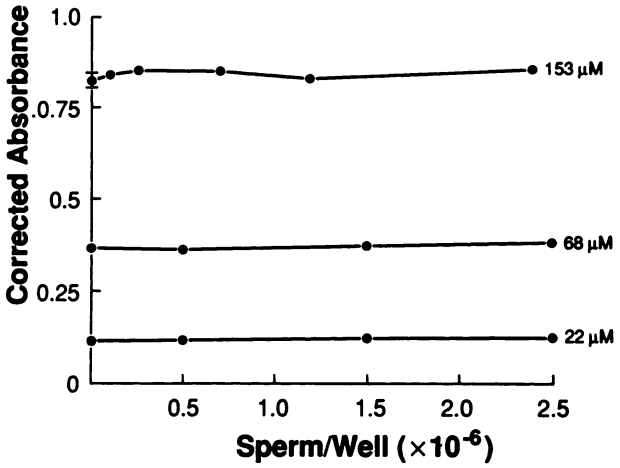

Fig 1.—Accuracy of correcting the optical absorbance for the presence of sperm. Wells contained a solution of PNA (either 22, 68, or 153 μmol/L). Additionally, wells contained from 0 to 2.5×10^6 sperm. The absorbance was determined in the dual wavelength mode, and further corrected by subtracting the absorbance of a control solution containing an equal number of sperm. Each point is the average of two or three replicates. Bars denote SE; where no bars are shown they would be shorter than the width of the symbol. The addition of sperm to the wells had little effect on the corrected absorbance.

Discussion

The modified assay described here significantly shortens and simplifies the assay of Kennedy et al (1989), making it more suitable for use in a clinical setting. The results obtained with the new protocol were not significantly different from those obtained using the parent assay. Because Kennedy et al (1989) showed that their assay gives the same results as measuring the extractable acrosin activity, it is reasonable to conclude that the new protocol described here also gives the same results as assaying extractable acrosin.

The procedure for preparing the sperm for the assay is somewhat different from that devised by Kennedy et al (1989). In their assay, a known number of sperm is layered onto the Ficoll solution, pelleted by centrifugation, resuspended, and assayed. In preliminary experiments, significant numbers of sperm were sometimes lost during

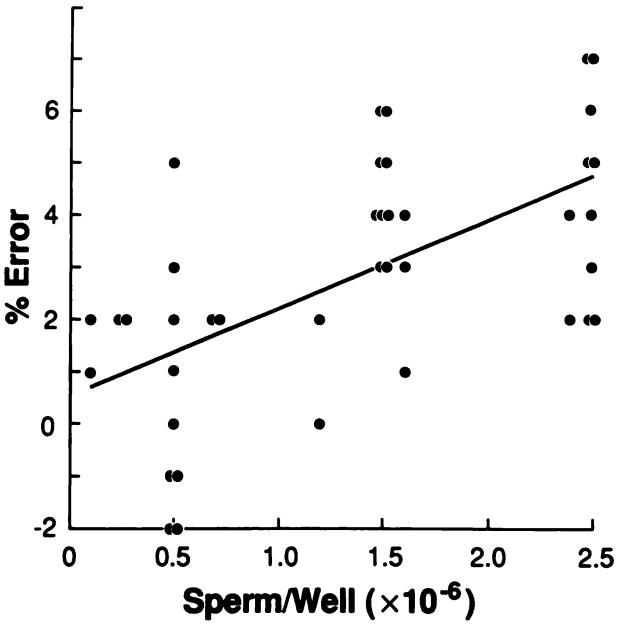

Fig 2.—Accuracy in the procedure that corrects the absorbance for the presence of sperm. The data in Fig 1 were recalculated as a percent error (100% × [corrected absorbance in the presence of sperm/corrected absorbance in the absence of sperm]). Each point is a single replicate.

centrifugation (data not shown), and it seemed more prudent to count the sperm and adjust the concentration after centrifugation. The sperm concentration should be determined carefully, using standard laboratory procedures (eg, Mortimer et al, 1986), because the sperm number determines the denominator of the specific activity.

It is recommended that 1.5×10^6 sperm be assayed per well. This number of sperm produces a

Table 1. *Comparison of the results of the new assay with results of a conventional assay (see Material and Methods)*

Sample	Acrosin activity (μIU/10⁶ sperm; average ± SD [n]) Conventional assay	Simplified assay
A	29.6 ± 0.4 (2)	28.4 ± 0 (2)
B	37.1 ± 0.4 (2)	37.9 ± 0 (2)
C	61.1 ± 1.6 (2)	63.4 ± 15.7 (6)
D	74.1 ± 0.8 (3)	73.0 ± 6.0 (8)
E	61.3 ± 1.8 (2)	68.0 ± 2.1 (5)
F	111.0 ± 4.2 (2)	101.0 ± 3.7 (3)
G	39.6 ± 1.1 (2)	41.2 ± 2.6 (3)
H	28.0 ± 0.8 (2)	32.3 ± 0.4 (4)
I	20.6 ± 0.6 (2)	25.6 ± 2.0 (4)
J	22.8 ± 0.9 (2)	26.4 ± 2.0 (5)
K	25.8 ± 0.1 (2)	31.2 ± 0.9 (5)

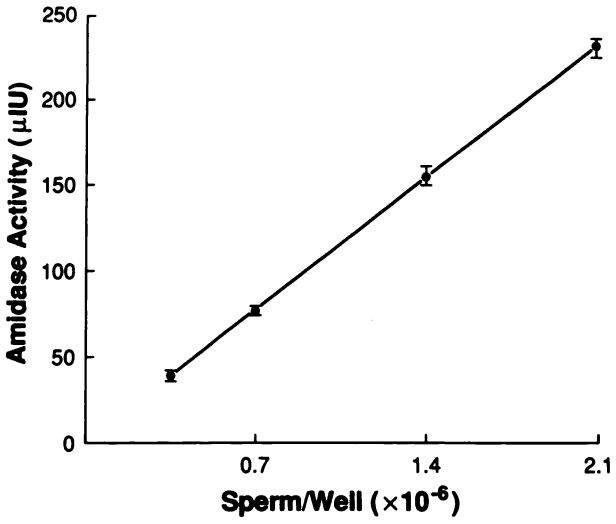

Fig 3.—Relationship of measured enzyme activity to the number of sperm present. Bars denote SE.

suitable amount of reaction product for almost all samples. The calculated sensitivity of the assay is 7.6 μIU/well. This limit will not often be reached if 1.5×10^6 sperm/well are used. (The activities of 35 semen samples assayed by Kennedy et al (1989), ranged from 7 to 60 μIU/10^6 sperm.) Activity in the lowest range should be detectable, but accurate measurement would require the use of more sperm or a different assay.

Time is saved in the new protocol in several ways. Partly it is the result of using fast hardware and software: the microplate reader measures and calculates the difference absorbance of 96 samples in 1 minute, and computer-aided data capture and calculation can be used to carry out the remaining data manipulation. The assay is also considerably shortened by not centrifuging the sperm out of the suspensions before determining the absorbance. The absorbance caused by the presence of sperm is corrected by measuring in the dual wavelength mode and by subtracting the absorbance of the control wells from that of the test wells. These corrections leave a very small residual sperm-associated absorbance, which increases the apparent absorbance of the reaction product by about 3%. If this level of error is unacceptable, or if it is necessary to use a higher sperm concentration that substantially increases the error, then a different assay should be used. Time is also saved by not adding benzamidine to the blank and test wells. If the absorbances are read at exactly 3 hours, it is not necessary to stop the reaction. Alternatively, ben-

zamidine can be added at 3 hours to terminate the reaction if the plates are to be read later.

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