

# Isolation and Purification of Acrosin (ACR) from the Human Seminal Fluid of Infertility Patients

Khalis Mohammed Shahadha<sup>1</sup>, Shihab Ahmed Al-Bajari<sup>2</sup>, Firas Tahir Maher<sup>3</sup>

<sup>1</sup>Department of Chemistry and Biochemistry, College of Medicine, Nineveh University, Mosul, Iraq, <sup>2</sup>College of Agricultural Technology, Northern Technical University, Mosul, Iraq <sup>3</sup>Department of Chemistry, College of Science, Tikrit University, Tikrit, Iraq

## Abstract

**Background:** Acrosin (ACR) is one of the most important proteases in the mammalian sperm head, located within the acrosome of human spermatozoa. ACR is a proteolytic enzyme that may hydrolyze the zona pellucida in oocytes. Following its release from the acrosome, it attaches to the zona pellucida of the egg and creates small openings in the zona pellucida, allowing the sperm to enter and fertilize the egg. **Objective:** It was the isolation and purification of the ACR from a human seminal fluid of infertile patients and the study of the optimal conditions required for the partially purified enzyme. **Materials and Methods:** Include the isolation and purification of the ACR from a human seminal fluid of infertile patients using a variety of biotechniques, such as ion-exchange chromatography, where one highly active primary peak is separated by the diethylaminoethyl cellulose (DEAE-C) ion-exchange technique, dialysis, and sedimentation with 65% ammonium sulfate. **Results:** A major protein bundle was separated with strong activity for the ACR with a 58% recovery rate of the enzyme. optimal conditions determined for the partially purified enzyme, the highest activity was for reaction time at (9 min), (pH = 7.5), enzyme concentration (500 ng/mL), substrate concentration (60 mmol/L), temperature (37°C),  $V_{max}$  (21.23  $\mu$ mol/min), and  $K_m$  (19.12 mmol/L), respectively, using the Lineweaver–Burk plot. Molecular weight of ACR partially purified was estimated a 66-kDa by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique. **Conclusion:** Low levels of ACR appear to be linked to infertility and subfertility. Thus, measuring ACR activity is a viable method for assessing the fertilizing potential of human spermatozoa.

**Keywords:** ACR, acrosin, ion exchange, isolation, maximum velocity ( $V_{max}$ ), Michaelis–Menten constant ( $K_m$ ), purification, SDS-PAGE technique

## INTRODUCTION

Male infertility occurs when a male partner is unable to get a woman pregnant after a year of frequent, unprotected sexual activity. Disorders in sperm production account for 30% to 50% of cases of male infertility.<sup>[1]</sup> Pregnancy is achieved when the male sperm cell successfully connects with and fertilizes the female ovum. Pregnancy results from the embryo or fetus's cellular development.<sup>[2]</sup> Male infertility may result from too low sperm production, an abnormally shaped sperm, insufficient motility, or a problem with the delivery system that prevents moving sperm from reaching the target. Chronic injuries and an improper lifestyle can affect male fertility.<sup>[3]</sup> As a result of medical or immunological factors or environmental exposures,<sup>[4]</sup> diagnosis of male infertility needs to be performed

with variety of tests on the patient before beginning the treatment, like a General medical examination, Semen Analysis, Testicular biopsy, Endocrine evaluations, Post-coital test, Examination of split ejaculation, Sperm cross penetration test, Measurements of anti-sperm antibodies, Biochemical tests, and Identification of gene mutations that may cause infertility.<sup>[5]</sup> Sperm consists of a head, a mid-piece (mid-segment), and a tail. It is characterized by a minimal amount of cytoplasm, and the head contains a nucleus. Its shape is flat and oval, attaches to the egg and

**Address for correspondence:** Dr. Khalis Mohammed Shahadha,  
Department of Chemistry and Biochemistry,  
College of Medicine, Nineveh University, Mosul, Ninevah, Iraq.  
E-mail: khalis.mohammed@uoninevah.edu.iq

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penetrates it easily.<sup>[6]</sup> The anterior apex of the head of the sperm bears a cap-like structure called the acrosome and contains hydrolytic enzymes. Acrosin (ACR) is one of the most important proteases in the mammalian sperm head, located within the acrosome of human spermatozoa.<sup>[7]</sup> Acrosin is a proteolytic enzyme that may hydrolyze the zona pellucida (ZP) in oocytes, and its absence or diminished activity may be a contributing factor to male infertility. Thus, measuring ACR activity is an appropriate method for assessing the fertilizing potential of human spermatozoa. To test human sperm ACR activity, many procedures, including gelatinolytic, have been described.<sup>[8,9]</sup> Low levels of ACR appear to be linked to infertility and subfertility. Acrosin activity is unaffected by typical sperm characteristics such as density, percent motility, and morphology. Its absence or diminished activity may be a clinical cause of male infertility. Thus, measuring ACR activity is a vital method for assessing the fertilizing potential of human spermatozoa.<sup>[10]</sup> In most placental mammals, fertilization is a well-planned process involving a number of activities, such as a) penetration of the cumulus cell layer by sperm; b) sperm adhesion to the ZP; c) corpuscular response; d) ZP oviposition; and e) cell membrane fusion (sperm and ova).<sup>[11]</sup> Figure 1 shows the mechanism of action of ACR.<sup>[12,13]</sup>

## MATERIALS AND METHODS

This study included the collection of 108 semen samples from couples with idiopathic infertility. The samples

were collected from the patients at Al-Jamhuri Teaching Hospital in Mosul to be the study sample, according to the instructions of the supervisor. Specialist in urosurgery and infertile in the consultation of the Teaching Hospital in the city of Mosul, after obtaining the moral license from the patients and conforming to the controls of the Health Department in Nineveh Governorate.

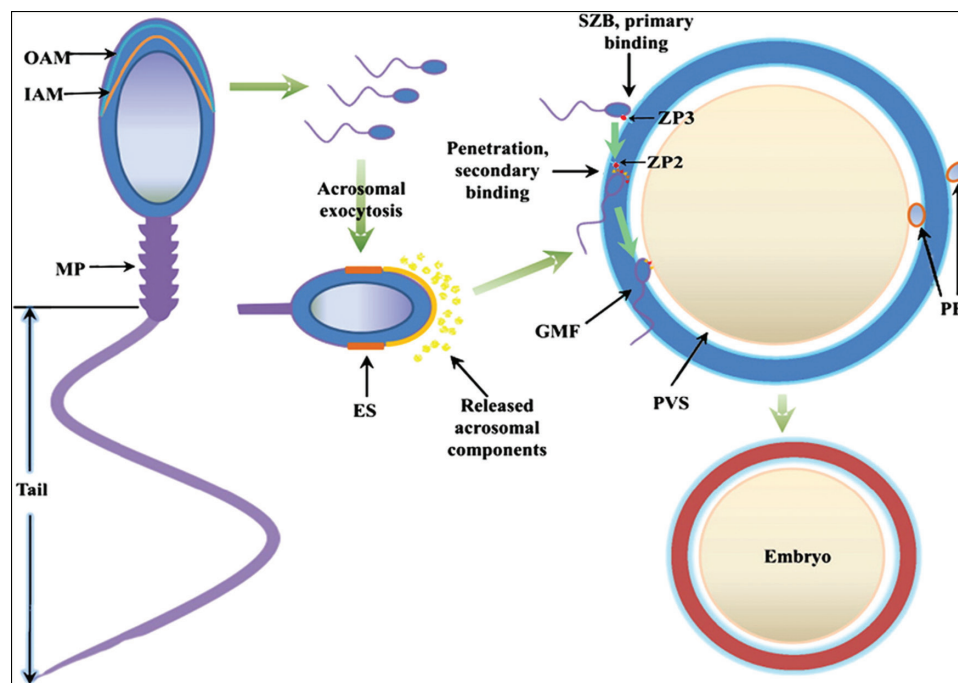
### Specimen used

Semen samples were collected from unexplained infertile donors with no chronic disease and nonsmokers. Their ages ranged between 22 and 47 years. After confirming the result of the SFA, the samples were collected in a sterile and dry tube, and the seminal plasma was separated by using a cooled centrifuge at a speed of 6000 xg for 30 min.<sup>[14]</sup> The seminal plasma will undergo a series of separation processes and purification stages necessary to separate and study the enzyme properties using several biological techniques, which will be explained below.

### Determination of human ACR enzyme in seminal plasma by the ELISA method

#### Principle of the assay

The principle for the kit used in determining ACR is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with human (ACR) antibodies



**Figure 1:** Sperm–egg interaction. During SZB and penetration, sperm–ZP primary and secondary binding are shown. SZB: sperm–ZP binding; ES: equatorial segment; OAM: outer acrosomal membrane; GMF: gamete membrane fusion; IAM: inner acrosomal membrane; PB: polar body; MP: middle piece; PVS: perivitelline space<sup>[12]</sup>

(ACR) present in the samples, which are added and bind to the antibodies coated on the wells. Then biotinylated (ACR) antibodies were added and bound to ACR in the sample. Then Streptavidin–HRP is added and binds to the biotinylated (ACR) antibodies. After incubation, unbound Streptavidin–HRP has washed away during a washing step. The substrate solution is then added, and color develops in proportion to the amount of human (ACR).<sup>[15]</sup> The reaction terminates with the addition of an acidic stop solution, and absorbance is measured at 450 nm.

### Assay procedure

Acrosin enzyme levels were determined in the seminal plasma using ELISA kits supplied by Fine Biotech Co, Ltd. (Wuhan, China) According to the assay procedure kits,<sup>[15]</sup> the male participants were divided into three groups.

1. Control group.
2. Infertile male due to impairment in semen parameters.
3. Male partner of couples with unexplained infertility.

### Calculation of result

Plot the best-fit curve through the points on the graph by drawing the average OD for each standard on the vertical (absorption) axis against the concentration on the horizontal (concentration) axis, as shown in Figure 2.

### Isolation and purification of ACR enzymes from seminal fluid

The following processes were used to purify the ACR from male infertility patients' blood serum using customary purification techniques:

#### Precipitation of enzymes with ammonium sulfate

This process is summarized by adding inorganic salts such as ammonium sulfate, which causes large amounts of water molecules to leave the protein and bind to the salt

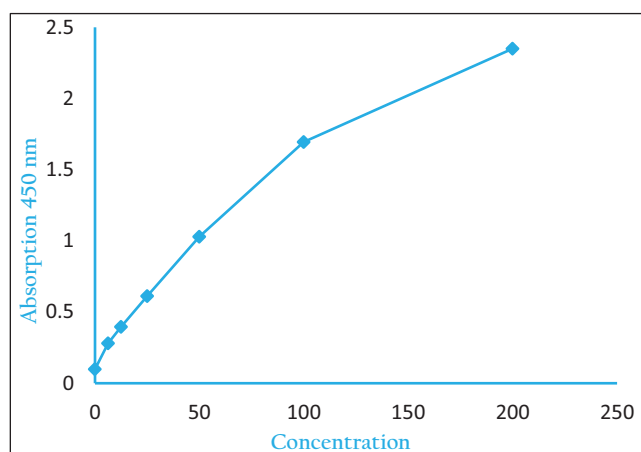


Figure 2: Standard curve of the ACR

molecules because they are more soluble than the poorly soluble protein molecules, so the protein molecules clump together. Seminal plasma was used, and ammonium sulfate ( $(\text{NH}_4)_2\text{SO}_4$ ) was added to it in its solid state with a saturation ratio of 65% and gradually while stirring the mixture with a magnetic stirrer at  $-4^\circ\text{C}$  for a period of 60 min.<sup>[16]</sup> After leaving the mixture in the refrigerator for a period of 24 h, the precipitate was separated from the filtrate by using a cooled centrifuge at a speed of  $6000 \times g$  for 30 min. Then, the amount of protein was estimated by the modified Lowry method using ready solutions from the Chinese company Fine Biotech Co., Ltd. The activity of the ACR enzyme was measured for the protein precipitate.<sup>[12,13]</sup>

### Purification by dialysis

The membrane sorting process was carried out by placing the protein solution in a cellophane tube tightly tied from the bottom in advance, then tied tightly from the top with a string. Then the tube was placed in a volumetric vessel containing 3 L of ammonium bicarbonate solution ( $\text{NH}_4\text{HCO}_3$ ), (0.1M). The membrane sorting process was carried out at  $4^\circ\text{C}$  with continuous stirring with the magnetic stirrer, and the process lasted 24 h and was then left for the following day. The amount of protein was estimated using the modified Lowry method as well as measuring the activity of the enzyme ACR in the solution resulting from the membrane sorting.<sup>[17,18]</sup>

### Purification by diethyl amino ethyl cellulose ion-exchange chromatography

Diethyl amino ethyl cellulose (DEAE\_Cellulose 52) ion exchanger was prepared according to the instructions of the supplying company (Whatman, Wuhan, China), using a glass separation column with dimensions ( $2.5 \times 40\text{cm}$ ), and the column was filled with activated ion exchange resin by pouring it quietly on the walls of the column to prevent the formation of air bubbles.<sup>[19]</sup> Then leave it until the resin particles settle. Most of the excess buffer solution was withdrawn from the column, and then another amount of resin was added. This process was repeated until the resin level reached a height of 35 cm. Then the resin was allowed to compact by being allowed to agglutinate by passing (0.05 M) phosphate buffer ( $\text{pH} = 7.5$ ), allowing the buffer solution to descend from the bottom hole of the column. Then pass the sample resulting from the dialysis process. Injecting it into the top of the column quietly, then passing the phosphate buffer with a graded concentration from 0.05 to 0.3 M and ( $\text{pH} = 7.5$ ). Lyophilization was done using a lyophilizer, freezing the solution to be lyophilized in a plastic beaker by placing it in the freezer ( $-20^\circ\text{C}$ ), and then placing in the lyophilizer.<sup>[20]</sup>

### Determination of the molecular weight of the ACR by the SDS-PAGE technique

The researcher (Laemmler)<sup>[18]</sup> used the method to estimate the molecular weight of an enzyme, which includes the

addition of sodium dodecyl sulfate (SDS), which has the ability to separate the protein units into secondary units and give them a large number of negative charges, thus removing the original charge on the molecule and making the separation depend on its molecular weight. According to the procedure that was used, Laemmli prepared each of the stock solutions and working solutions.<sup>[14]</sup> To prepare a gel column, (3 mL) of the solution prepared in paragraph (2) was taken and placed in a plastic tube (12 cm) long and (0.5 cm) in diameter. Then the initial polyacrylamide gel was placed on top of the first gel in the plastic tube after pouring the previously placed water layer, as 0.5 mL of this solution was added on top of the separation gel layer, and the tubes were then placed near a fluorescent light source until the polymerization process. The sample was prepared by mixing 50  $\mu\text{L}$  of protein dissolved in solution (2) at a concentration of 2 mg/mL with 10  $\mu\text{L}$  of SDS (10%) solution (4) with 3  $\mu\text{L}$  of 2-mercaptoethanol, then heating the mixture in a boiling water bath for 3 min. Minutes later, an equal volume of 20% sucrose was added to the mixture, and 10  $\mu\text{L}$  of bromophenol blue dye at a concentration of 0.005%. Then 25  $\mu\text{L}$  of this solution was taken and placed on the surface of the gel after filling the separator reservoirs with solution (7) diluted by 1:10 with distilled water. Then the positive pole (Anode) was connected to the lower tank and the negative pole (Cathode) to the upper tank, and an electric current was connected at an amount of 80 mA and a voltage estimated at 150 V. Then the fixation process was carried out by immersing the gel columns in a solution (20%) of TCA for (20 min), after which they were washed with distilled water and immersed in Coomassie brilliant blue R125 dye. Then excess dye attached to the gel was removed only by immersing the gel columns for 48 h in the dye removal solution.<sup>[21]</sup>

### Protein estimation using the modified Lowry method

Protein was estimated using the modified Lowry method. The intensity of the resulting color depends on the protein content of the amino acids tyrosine and tryptophan. Preparation of each alkaline and fallen's reagent. Bovine serum albumin was used as a standard solution to prepare the standard curve for protein which has a molar absorption coefficient equal to  $(0.67 \text{ M}^{-1} \text{ cm}^{-1})$ .<sup>[22]</sup>

### Kinetic analysis of a purified ACR enzyme

#### *Effect of the enzyme concentration on activity (ACR)*

The presence of different quantities (50–500 ng/mL) of the partially purified enzyme was used to measure ACR activity.

#### *Effect of temperature on activity (ACR)*

The enzyme activity was measured at different temperatures ( $7^{\circ}\text{C}$ – $61^{\circ}\text{C}$ ) to determine the optimum temperature that gave the highest enzyme activity.

### *The influence of pH on ACR activity*

Acrosin activity was tested in a buffer solution with varied pH values in the range of 4–10 (pH) to determine the maximum enzyme activity.

### *The influence of incubation time on ACR activity*

The optimal timing for maximizing enzyme activity was determined by looking at the reaction time. The enzyme activity was checked every 3 to 30 min.

### *Effect of substrate—Concentrations of ACR activity*

To study the effect of the substrate on the activity of the partially purified ACR, different concentrations of N-benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA) were prepared to determine the optimal concentration that results in the highest speed maximum velocity ( $V_{\text{max}}$ ) and Michaelis–Menten Constant ( $K_m$ ).

### Statistical analysis

In order to calculate the mean and standard deviation and evaluate if there were any significant differences between the research groups, the laboratory test data were analyzed using a one-way Analysis of Variance (ANOVA) and a *t* test in the program SPSS (version 16, Armonk, New York: IBM Corp). When using the  $P < 0.01$ , it means a significant difference at the 1% probability level;  $P = 0.01$ – $0.05$ , it means a significant difference at the 5% probability level;  $P > 0.05$ , no significant differences between the probabilities.

### Ethical approval

The study was conducted in accordance with the ethical principles that have their origins in the Declaration of Helsinki. It was carried out with patients' verbal and analytical approval before the sample was taken. Patients were informed of the study's goal before providing their consent, and those who refused to participate in the study were excluded. Scientific and ethical approval has been obtained for the isolation and purification of ACR from the human seminal fluid of infertility patients in accordance with our institutional research committee's ethical guidelines according to document approval number 3/7/933 dated March 15, 2023.

### RESULT

The study included 180 patients, whose ages ranged from 22 to 27 years. They were divided into three groups:

- A: 65 infertile men with reason.
- B: 65 infertile men without reason.
- C: 50 fertile men as a control group

### Acrosin enzyme activity in human seminal fluid

The enzyme activity of ACR was measured and compared in a human seminal fluid from patients who were infertile

males, patients who had unexplained infertility, and the control group. Using the method followed by the researcher, the results indicated in Table 1 show the average activity of the ACR enzyme in the seminal fluid of patients who have infertility with reason, patients who have infertility without reason, and those in the control group. The results showed that there was a significant difference in the level of probability ( $P \leq 0.01$ ) for patients who have infertility with a reason ( $5.6733a \pm 0.0074$ ) and patients who have infertility without reason ( $8.3164b \pm 0.4406$ ) compared with the control group ( $11.7544c \pm 0.2433$ ) [Table 2].

**Characterization of optimal conditions for purified ACR activity from the human seminal fluid of infertility patients**

*Effect of the enzyme concentration on activity (ACR)*

Acrosin enzyme activity was measured using different concentrations of the partially purified enzyme from the human seminal fluid of donors who were patients infertile without reason, ranging (50–500 ng/mL) from the solution of the protein package, as it was observed that the enzyme reaction velocity will increase with the increase of the enzyme concentration, as shown in Figure 3. That is, the rate of the enzyme catalyzed reaction is directly proportional to the concentration of the enzyme when the substrate is available in the environment.

*Effect of temperature on activity (ACR)*

The activity of the ACR was measured at different temperatures ranging from 7°C, 13°C, 19°C, 25°C, 31°C, 37°C, 43°C, 49°C, 55°C, and 61°C, and it was noted in Figure 4 that the gradual rise in temperature leads to an increase in the speed of the enzymatic reaction, as it

appeared that the optimum temperature (37°C) shows a maximum of ACR activity of 17.1 U/mL. And after that, the activity of the enzyme decreased significantly because the enzymes have a protein nature and are sensitive to temperature changes. This may cause denaturation of the enzyme due to the breaking of the hydrogen bonds and the other force responsible for maintaining a three-dimensional structure and thus the loss of the enzyme’s effectiveness.

*The influence of pH on ACR activity*

The activity of the enzyme was measured by changing the pH of the phosphate buffer solution in the range of 4–10 pH of the same concentration, and the results shown in Figure 5 depict the influence of varying degrees of pH (4–10) on ACR activity, which increases with rising pH until it achieves optimal (pH = 7.5) to determine the maximum enzyme activity. Since each enzyme has an acidic function, the enzyme shows its highest activity, which is called the optimal pH, and using a high or very low pH will lead to the loss of the enzyme’s effectiveness as a result of the occurrence of denaturation or distortions in the nature of the enzyme.<sup>[23]</sup> Show it that the pH of an enzymatic process has a significant impact on how quickly it reacts.

*The influence of incubation time on ACR activity*

The activity of the enzyme was measured at different times from the start of the reaction to establish the optimal timeframe for providing the enzyme’s maximal activity. The results shown in Figure 6 indicate that the best activity of the enzyme was at 9th min and then the activity begins to decrease due to a decrease in the concentration of the substrate or the similarity of the active site to the substrate.

**Effect of substrate concentrations on ACR activity, and calculating the Michaelis–Menten constant ( $K_m$ ) and the maximum velocity ( $V_{max}$ )**

The activity of the ACR was measured in the presence of different concentrations of N-benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA) on the ACR (0, 5, 15, 25, 35, 45, 50, 60, 70, 80, 90 µg/mL) as a substrate. It was found that the maximum activity of the ACR was obtained by using 60 µg/mL of BAPNA, Figure 7 shows that the reaction rate of the ACR will increase with increasing concentrations of substrate

**Table 1: Acrosin enzyme activity in a human seminal fluid**

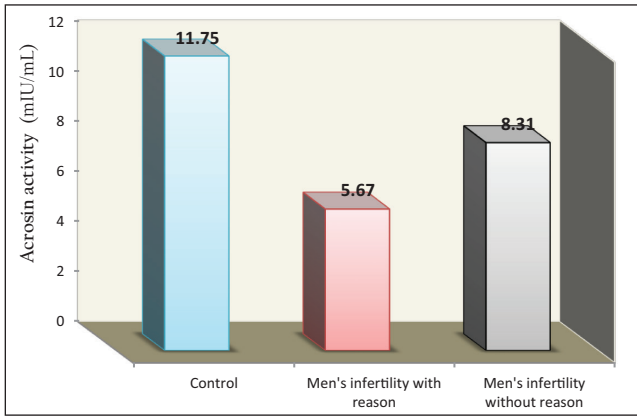
Groups	No.	Mean	± SD
Men infertility with reason	65	5.6733 a	0.0074
Men infertility without reason	65	8.3164 b	0.4406
Control	50	11.7544 c	0.2433
P value	0.000**		

\*\*significant difference at the probability level of ( $p \leq 0.01$ ), according to t test.

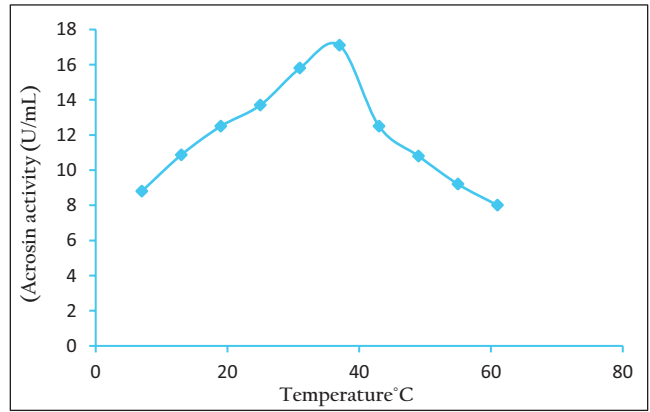
**Table 2 : Purification steps for ACR from the seminal fluid of patients who have infertility without reason**

Purification steps	Volume (mL)	Protein concentration (mg/mL)	Total protein (mg)	Enzyme activity (U/m × 10 <sup>-3</sup> )	Total activity (U × 10 <sup>-3</sup> )	Specific activity (U/mg)	Purification (Folds)	Yield (%)
Crude enzyme	17	5.1	86.7	17.5	297.5	3.43	–	–
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation: 65%	8.4	4.7	39.48	17.8	149.52	3.78	1.1	50.25
Dialysis	11.3	4.3	48.59	18.1	204.53	4.2	1.22	68.74
Ion exchange	25	1	25	1.21	30.25	7	2.04	58.82

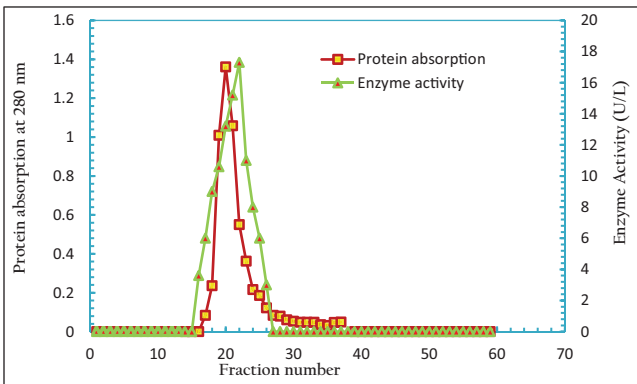
\*Enzyme unit is the quantity of ACR in nanograms (ng) that transforms the substrate into product per minute/milliliter under the given measurement circumstances



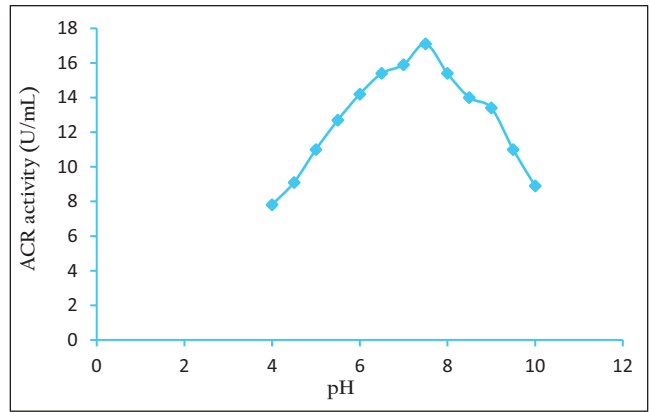
**Figure 3:** Effect of the ACR concentration on the enzyme reaction velocity



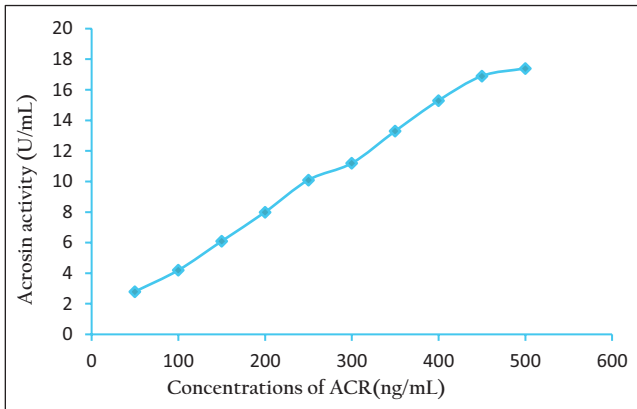
**Figure 6:** Effect of the incubation period on ACR activity



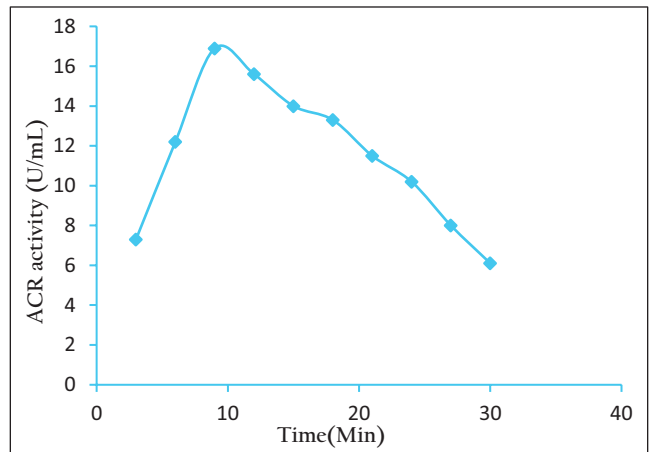
**Figure 4:** Effect of temperature on activity ACR



**Figure 7:** Effect of different concentrations of the substrate on the activity of the ACR enzyme



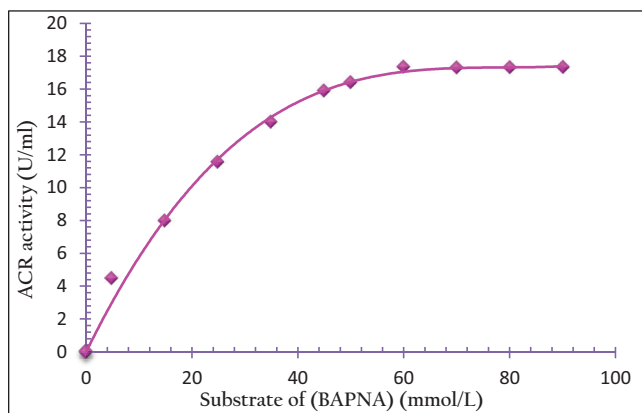
**Figure 5:** The influence of pH on ACR activity



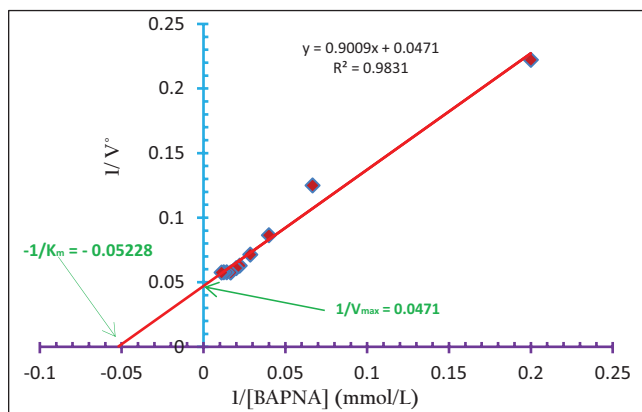
**Figure 8:** ACR enzyme activity in study groups (mIU/mL)

until it reaches a constant value, after which there is no increase in the rate of the enzymatic reaction rate with an increase in the concentration of BAPNA until it reaches its highest maximum velocity ( $V_{max}$ ) at the optimal concentration. As shown in Figures 3, 4, 8, and 9, the saturation of the ACR enzyme with the N-benzoyl-DL-arginine p-nitroanilide hydrochloride was at a concentration of 17.34 U/mL. The findings indicated using the Lineweaver-Burk plot, find the

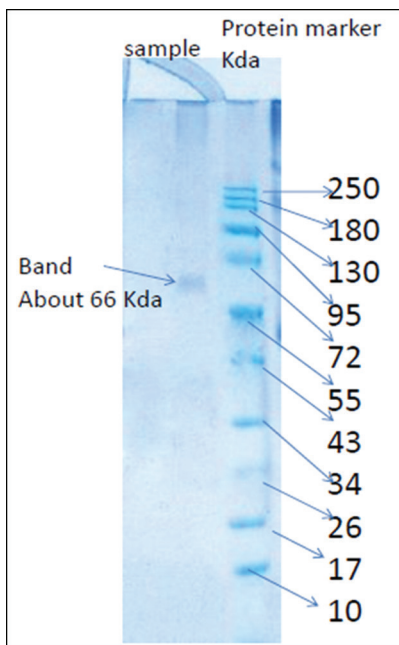
value of the maximum speed (21.23) at 60  $\mu\text{g/mL}$  and the Michaelis-Menten constant ( $K_m$ ) (19.12), respectively, as shown in Figure 10.  $K_m$  is an essential factor in enzyme kinetics;  $K_m$  is the (BAPNA) level that yields ( $1/2 V_{max}$ ) and so serves as an indicator of the ACR's affinity for the (BAPNA). Acrosin with a high affinity for its (BAPNA) has a low ( $K_m$ ) value; the ( $K_m$ )



**Figure 9:** Profile of the ACR proteinous peak from the seminal fluid of male patients who were infertile without reason using the DEAE-cellulose 52 ion-exchange column



**Figure 10:** Lineweaver-Burk plot to show the values of the Michaelis-Menten constant and the maximum velocity



**Figure 11:** SDS-PAGE analysis bands of the purified ACR and the standard protein markers

value estimates the intercellular level of the (BAPNA), whereas ( $V_{max}$ ) indicates the amount of active ACR present.

**Determination of the approximate molecular weight of partial purified ACR using SDS-PAGE electrophoresis**

The molecular weight of ACR, that is, partially purified from a human seminal fluid of infertility patients, was measured and ranging in age (22 to 47 years) by electrophoresis on SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) using a standard curve of the logarithm of molecular weight versus relative migration of 11 of standard protein markers between 10 and 250kDa. When the enzyme is treated with SDS, it disassembles the protein, giving chains of variable sizes surrounded by negatively charged SDS molecules. As it removes the original charge of the enzyme, and these chains move by electrical migration. The distance traveled by proteins to the anode depends on the ratio of the charge to the mass. The movement of protein markers, as mentioned previously, during electrical migration depends on several factors, including the molecular weight, when other factors such as electric current and charge are fixed. Protein markers will move at different distances depending on their molecular weight.<sup>[18]</sup> As shown in Figure 11 below, the molecular weight was calculated by estimating approximately 66kDa for ACR, resulting from a comparison between the enzyme bundle and standard bundle using the standard curve shown in Table 3 and Figure 12.

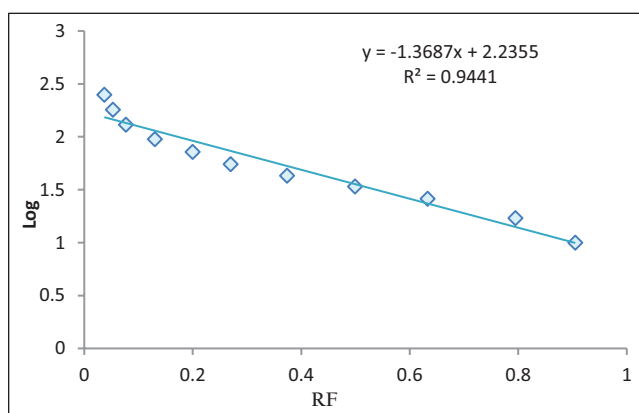
**DISCUSSION**

**Acrosin enzyme activity in the human seminal fluid**

The results indicate in Table 1 and Figure 8 the different levels of ACR activity in a human seminal fluid. It showed that there was a significant difference in the level of probability ( $P \leq 0.01$ ) for patients who were infertile with a reason ( $5.6733a \pm 0.0074$ ) and patients infertile without reason ( $8.3164b \pm 0.4406$ ) compared with a control group ( $11.7544c \pm 0.2433$ ). Because of several factors that can contribute to this variation, ACR levels or activities in seminal fluid might fluctuate due to various genetic differences.<sup>[24]</sup> Age and medications may also affect the ACR activity of the seminal fluid.<sup>[25]</sup> According to studies, drinking alcohol in moderation may enhance ACR activity in seminal fluid, whereas smoking may decrease it. Also, ACR activity in the seminal fluid may be impacted by infections of the reproductive system, including prostatitis and epididymitis.<sup>[26]</sup> Acrosin activity can be decreased by inflammatory circumstances.<sup>[27]</sup> There are many studies conducted about ACR activity in human seminal plasma and cervical mucus samples from fertile and infertile men. The researchers found that ACR activity was significantly higher in the seminal

**Table 3: Protein markers used in the determination of the molecular weight of ACR by SDS-PAGE**

Band	MW	Log	Cm	RF
Myosin	250	2.39794	0.8	0.037
$\beta$ -Galactosidase	180	2.2552725	1.2	0.053
Bovine serum albumin	130	2.1139434	1.4	0.077
Phosphorylase	95	1.9777236	1.9	0.13
BSA	72	1.8573325	2.8	0.2
Glutamic dehydrogenase	55	1.7403627	4	0.27
Ovalbumin	43	1.6334685	4.8	0.374
Carbonic anhydrase	34	1.5314789	5.4	0.499
Myoglobin	26	1.4149733	6.1	0.633
Lysozyme	17	1.2304489	7.1	0.795
Cytochrome c	10	1	8.5	0.905

**Figure 12:** Determination of molecular weight of partial purified ACR by SDS-PAGE technique

plasma of fertile men compared to infertile men. This study is consistent with our current study. Another study compared the ACR activity in normal sperm and asthenozoospermic men. The results showed that asthenozoospermic men had significantly lower ACR activity compared to normozoospermic men. This is evidence of a positive correlation between ACR activity and sperm motility.<sup>[28]</sup>

### Characterization of optimal conditions for purified ACR activity from the human seminal fluid of infertility patients

Purification of ACR from seminal fluid is typically necessary before conducting detailed studies. This involves separating the enzyme from other components present in the seminal fluid, such as proteins, lipids, and cellular debris. Various techniques, such as chromatography, may be employed to achieve high purity. Results show the estimation of optimal conditions for measuring the effectiveness of ACR partially purified from the seminal fluid of infertility patients. It had an ACR concentration of 500 ng/mL with a pH of 7.5 and a reaction time of 9 min, a temperature of 37 C, a substrate concentration of 60 mmol/L, a Michaelis–Menten constant ( $K_m$ ) of 19.12,

and a maximum velocity ( $V_{max}$ ) of 21.23. Investigating the effects of these factors can help elucidate the regulatory mechanisms of ACR and its functional significance in infertility. Stability studies can provide guidelines for storing and handling purified ACR to ensure its integrity during subsequent experiments. Characterizing the optimal pH range and temperature for ACR activity can provide insights into its stability and functionality. Also, selecting an appropriate substrate is crucial for accurately measuring ACR activity. By characterizing the optimal conditions for purified ACR activity in the seminal fluid of infertility patients, researchers can gain insights into the enzyme's functionality and its potential role in male infertility. This information can contribute to a better understanding of the underlying mechanisms of infertility and potentially guide the development of diagnostic or therapeutic approaches targeting ACR or related factors.

Purification of the ACR is critical for getting a sounder understanding of how the enzyme functions. It is also important for investigating properties and better understanding structure–function relationships. In our investigation, ACR was isolated from the human seminal fluid of infertile patients 11 and 25X using dialysis-based precipitation and DEAE-cellulose ion-exchange methods, respectively. It has been demonstrated that ACR is active throughout a wide pH range. Acrosin from human sperm was shown to be active in pH ranges between 4.0 and 10.0, with optimum activity at pH 7.5. According to different research, the ACR from pig sperm was still active within the pH ranges of 5.0–10.0, with (7.5) being the ideal pH for activity, agreeing with our result.<sup>[26]</sup> It was also proven that the effectiveness of ACR is affected by changing the temperature, and it was proven during our study that the highest activity of the enzyme was at 37°C, and this corresponds to studies it has been noted that human ACR functions best at a temperature of around 37°C–40°C, which is also the average body temperature of people.<sup>[11]</sup> The purified ACR has a  $K_m$  and  $V_{max}$  of 19.12 mmol/L and 21.23  $\mu$ mol/min, respectively. Also, the current study's findings reveal that ammonium sulfate precipitation, ion exchange, and SDS-PAGE electrophoresis all result in a considerable increase in ACR-specific activity.

### DEAE-cellulose 52 ion-exchange chromatography

The ion-exchange chromatography technique was used to purify ACR after the dialysis method to separate proteins according to charge.<sup>[29]</sup> If the protein solution resulting from the dialysis process was passed on the separation column containing the negative ion DEAE\_Cellulose 52. Results showed in Figure 9 that there was one enzyme peak that appeared in the washing step, and one protein peak appeared after elution by the gradient concentrations of sodium chloride. If the specific activity of the protein reached 7.0 U/mg and the elution volume was 16–27 mL, this means The number of purification times doubled

after the ion exchange process by (2.04) and the amount of recovery of the total activity was (58.82%) more than it is in the raw semen.

The analysis of purified ACR using gel polyacrylamide electrophoresis with SDS revealed that there was no detectable contamination, as it represented just one distinct band, and the molecular weight of the enzyme was about 66kDa shown in Figure 11. The presence of several protein bands on the gel is attributed to the crude extract, which contains a diverse range of mixed proteins (protein markers) that have varying molecular weights. The purified ACR enzyme sample gave only one band, which means there is no contamination from those protein markers.<sup>[30]</sup> Also, prior research confirmed our finding and discovered that pure ACR delivers at 66kDa.<sup>[31]</sup> And our findings concur with MW Log Cm RF detection of pure ACR from a different source, giving 70kDa.<sup>[32]</sup> Additionally, other studies showed differences from our result and detected that purified ACR gives at 43kDa.<sup>[33]</sup> Acrosin's molecular weight can differ depending on whether it is in its active or inactive form, as well as whether it is in its precursor form (proacrosin) or its mature form (acrosin), it is important to note.<sup>[34,35]</sup>

## CONCLUSION

Low levels of ACR appear to be linked to infertility and subfertility. Acrosin activity is unaffected by typical sperm characteristics such as density, percent motility, and morphology. Its absence or diminished activity may be a clinical cause of male infertility. ACR isolation, purification, and molecular weight were estimated from the human seminal fluid of infertile patients using a variety of biotechniques. The study showed that there was a significant difference in the level of probability ( $P \leq 0.01$ ) for patients who were infertile with a reason ( $5.6733a \pm 0.0074$ ) and patients infertile without reason ( $8.3164b \pm 0.4406$ ) compared with a control group ( $11.7544c \pm 0.2433$ ). When comparing men with low sperm count (oligospermia) to those with unexplained infertility, both groups showed reduced ACR activity compared to the control group. However, it's important to note that unexplained infertility can encompass various underlying factors, and not all men with unexplained infertility will necessarily have a low sperm count. The specific causes and mechanisms of reduced ACR activity may vary in these cases.

## Conflicts of interest

There are no conflicts of interest.

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