Isolation and Purification of a Turkey Seminal Plasma Protease

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Abstract

A trypsin-like protease, distinct from spermatazoal acrosin,[1] was discovered in the seminal plasma of the domestic turkey, *Malleagris gallopavo*, and purified using preparative-scale isoelectric focusing in the Rotofor® cell. The turkey seminal plasma protease is being investigated as a possible plasminogen activator. Enzymes of this type are used as blood clotting agents.

After 5 hours in the Rotofor cell, as much as 70% of the protease activity and 71% of the total protein has been recovered from crude seminal plasma. There was approximately 5 fold purification of the protein of interest, with fractions between pH 7-8.5 containing ~50% of the protease activity and 10% of the total recovered protein. Purified material was dialyzed and lyophilized for studies of the enzyme's characteristics and kinetics.

Methods

Preparation of the Seminal Plasma for Isoelectric Focusing in the Rotofor Cell

Semen was collected from large white turkey toms and pooled. The semen was centrifuged three times at 12,800 g in a microcentrifuge, and the supernatant fluid was saved. The seminal plasma was filtered through a 0.45 µm membrane filter. An aliquot of this material was saved for an activity assay and protein concentration determinations (see Figure 1). The remaining seminal plasma (18.0 ml) was added to 2.5 ml Bio-Lyte® ampholytes (pH range 3-10, 40% w/v) and the volume brought to 50.0 ml with 1% glycine. This material was applied to the Rotofor cell without further treatment.

Running Conditions

The sample was focused for 5 hours at 12 W constant power. The voltage increased from 300 V initially to 690 V at equilibrium. Twenty fractions were harvested and their pH values measured. Each fraction was assayed for protein concentration with the Bio-Rad Protein Assay using bovine serum albumin (BSA) as the standard.

Protease Activity Assay

Activity of the protease in harvested Rotofor fractions was measured by using an N-α-benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA)-amidase assay.[2] The BAPNA-amidase reaction mixture consisted of 1.0 ml 0.25 M triethanoleamine buffer, pH 8.0, 0.5 ml 2.3 mM BAPNA, and 20-100 µl of sample at 25 °C. Absorbance at 405 nm was determined, and a molar extinction coefficient of 9,950 $M^{-1}cm^{-1}$ was used to calculate units of activity (µM BAPNA hydrolyzed/minute). Active fractions between pH 7-8.5 were pooled and dialyzed for 48 hours against three changes of distilled water to remove ampholytes. Samples were lyophilized, and the material was stored at -20 °C until reconstituted for further studies.

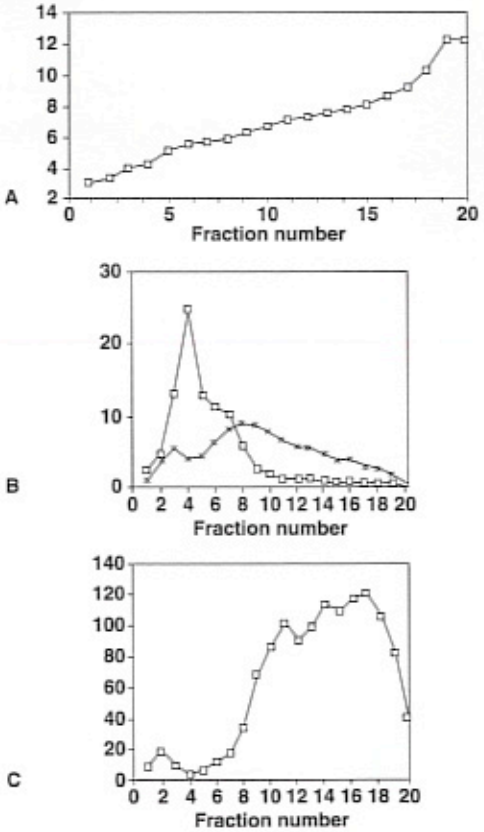

Fig. 1. Analysis of harvested Rotofor fractions. A. pH gradient. **B.** Percent protein recovered [×] (Bio-Rad Protein Assay) and percent recovery BAPNA-amidase activity [□]. **C.** Specific activity of the protease expressed as units/mg.

Native and SDS Electrophoretic Analysis

Rotofor fractions were analyzed using disc polyacrylamide gel electrophoresis at basic pH.[3] The native gel was stained with Coomassie blue G-250[4] which does not stain ampholytes (see Figure 2). Purified seminal protease, spermatazoal acrosin, and transferrin were analyzed by SDS-PAGE. The proteins were visualized by staining with Coomassie R-250 as shown in Figure 3.

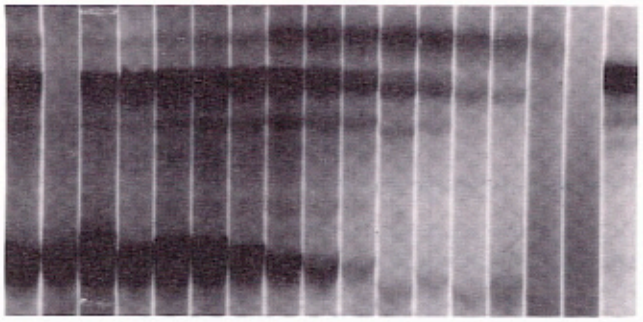

Fig. 2. Native gel of Rotofor purified seminal plasma. Lane 1. Crude turkey seminal plasma; **Lane 2.** Rotofor fractions 1-3; **Lane 3.** Fractions 4 and 5; **Lane 4-14.** Fractions 6-16; **Lane 15.** Fractions 17 and 18; **Lane 16.** Fractions 19 and 20; **Lane 17.** Turkey transferrin, purified from seminal plasma. Bands associated with enzyme activity are also seen in the transferrin standard indicating that the enzyme binds to transferrin.

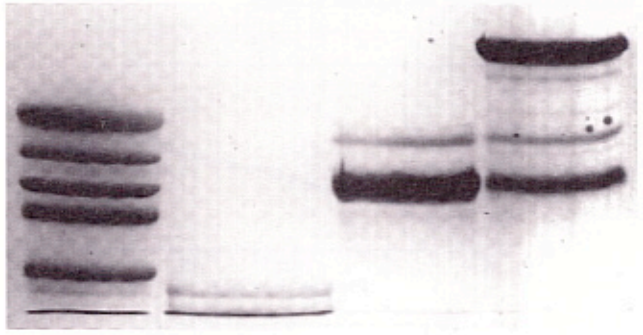

Fig. 3. SDS-PAGE analysis of purified seminal plasma protease. Lane 1. Molecular weight standards. **Lane 2.** Turkey sperm acrosin. **Lane 3.** Purified turkey seminal plasmas protease. **Lane 4.** Transferrin. Note that the enzyme subunits are also seen in the transferrin standards indicating that the enzyme also binds to transferrin. Molecular weights of standards from top to bottom: 45,000, 36,000, 29,000, 24,000, 20,100, and 14,200 kD.

Results

The crude seminal plasma contained ~390 units of BAPNA-amidase activity/ml and ~24.0 mg protein/ml; specific activity = 16.3 U/mg. Of the 6,427.5 units applied to the Rotofor cell, 66% (4,240.1 U) was recovered. Of 390 mg protein applied, 44% was recovered. Fractions 11-15 contained 59% of the BAPNA-amidase activity and 8% of the total recovered protein. This represented a 4-5 fold purification. Fractions 1-3 contained a white precipitate, and fractions 3-5 were yellow. All other fractions were clear and colorless. The precipitate in fractions 1-3 dissolved when pH was increased with 1.0 M NaOH. In other runs where less protein was applied, protein recovery has been as high as 70%.

The results of pH, protein, and BAPNA-amidase assays are summarized in Figure 1. The results of native electrophoresis of the Rotofor fractions revealed that fractions 13-18 consisted of three bands associated with enzyme activity (see Figure 2). Fractions 9-12 showed additional bands thought to be transferrin. Results of SDS-PAGE of pooled, active fractions showed that purified enzyme contained two bands (see Figure 3). The major band has an approximate molecular weight of 30,000 kD and the minor band has an approximate molecular weight between 36,000 and 45,000 kD. Both bands are also seen in the transferrin standard indicating that the protein binds to transferrin.

Conclusion

Preparative-scale isoelectric focusing in the Rotofor cell produced purification of a turkey seminal protease comparable with previous results obtained using gel-filtration column chromatography followed by DEAE-cellulose chromatography.[5] The ease and rapidity of purifying large quantities of protease in the Rotofor cell is unmatched by tedious chromatographic techniques. In the present study, 18.0 ml of crude turkey seminal plasma was used as starting material. Five fold purification of the protease was accomplished in less than 5 hours using the Rotofor cell.

References

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5. Thurston and Froman, unpublished data.

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