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A CATIONIC PROTEIN FROM A URATE-CALCIUM OXALATE STONE: ISOLATION AND PURIFICATION OF A SHARED PROTEIN

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Abstract

A protein extracted from a urate - calcium oxalate stone by electrodialysis is also excreted in the urine which served as the source material for its purification by FPLC after separation on an ACA44 column. It has an amino acid composition appropriate for a cationic protein. One peptide obtained by cyanogen bromide cleavage has significant (approximately 60%) homology with CD59 protein (protectin). Both proteins have wide distribution, the unknown having been found in bile, cholesterol gallstones, and the wall of the aorta. However, the two proteins appear to be immunologically different.

Key Words: Stone, urine, protein, CD59, SDS PAGE, immunoblot, amino acid, cationic, sequence.

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Introduction

Many proteins have been identified in renal stones since the pioneering work of Boyce *et al.* [2]. Some, like albumin and transferrin, are components of serum and urine, some are present in urine but absent or barely detectable in serum such as the Tamm Horsfall [3] protein and nephrocalcin [5], others have been extracted but not characterized and remain to be identified. This last group of proteins may be of special interest for their likely selective incorporation into stones would suggest an important role in the early events of stone formation. In order to study these early events *in vitro*, it is necessary to isolate and purify these proteins with minimal alteration of their native state. In this paper we report the purification and characterization of such a protein originally extracted from a urate-calcium oxalate renal stone and subsequently from urine.

Materials and Methods

This urate-calcium oxalate stone, the original source of the protein, was extracted as previously described by electrodialysis [1] in the ISCO apparatus and part of the extract served to develop an antiserum in the rabbit. This antiserum was used to scan concentrated urine and other extracts by immunodiffusion in agar. A urinary protein with reaction of identity with one of the stone proteins was detected, thus providing an unlimited quantity of material to isolate and purify the protein as follows: the urine is concentrated by dialysis against polyethylene glycol [PEG] and the proteins are precipitated by a finer PEG [molecular weight (M.W.) = 3,500 Daltons (D)] at a final concentration of 20%. The PEG is separated from the protein solution by the addition of 1 M sodium sulfate [4] and the lower phase is carefully removed and dialyzed in the cold room against large volumes of reverse osmosis distilled water for a minimum of 24 hours. The specimen is then concentrated against PEG and centrifuged in a refrigerated centrifuge. It is applied to a column (AcA44-Ultrogel-IBF, 10-130 kD M.W.) and the protein fractions eluted with 0.09 M Tris-Glycine buffer pH 8.2 at 4°C. The void volume is measured and the eluate is divided into three fractions

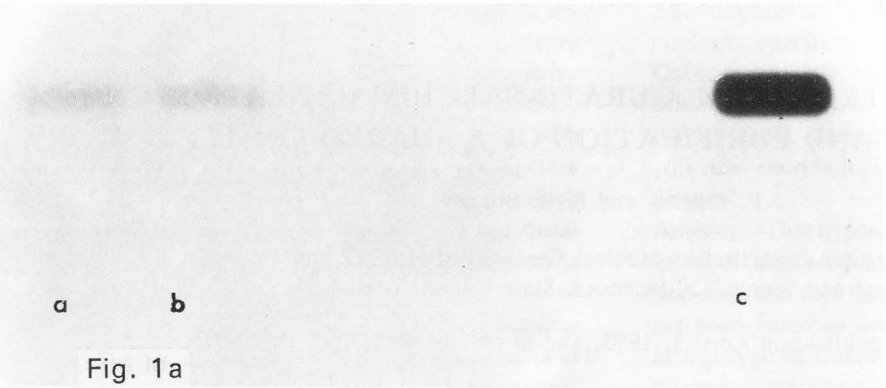

Fig. 1a

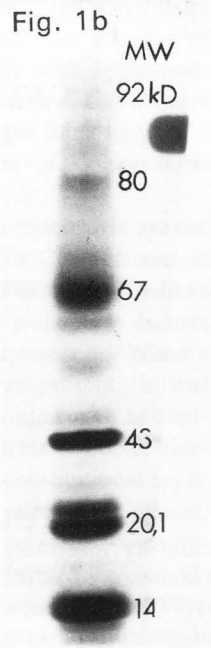

Fig. 1b

Figure 1a (above). SDS-PAGE electrophoresis. a and b represent 30 μl aliquots of the purified protein; c = 50 μl. The concentration of the specimen is 2 mg/ml. The stain is Coomassie.

Figure 1b (at right). Molecular weight standard from 20-92 kD; the concentration of unknown is 3-4 mg/ml and the quantity 40 μl. The stain is Coomassie.

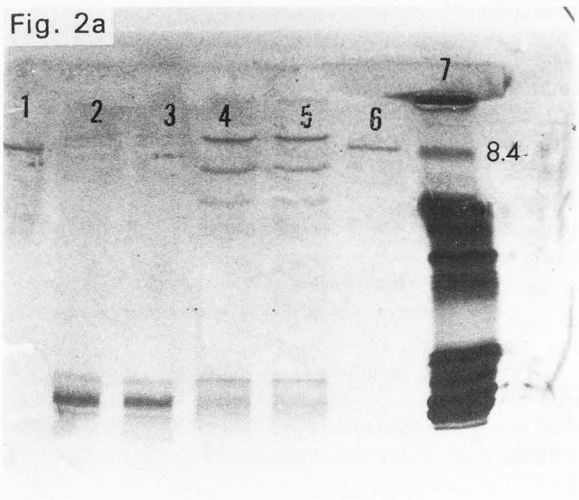

Fig. 2a

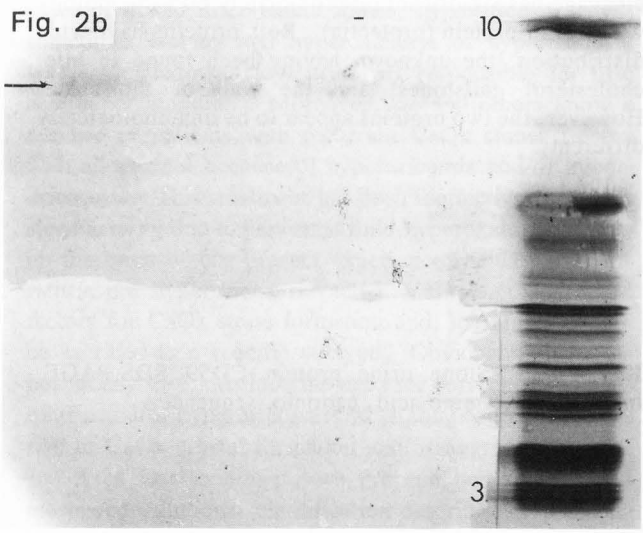

Fig. 2b

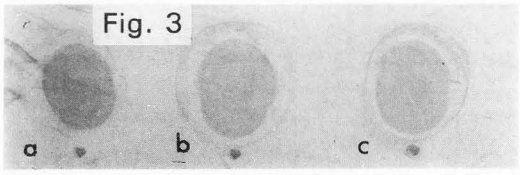

Fig. 3

Figure 3 (above). Immuno-dot of the purified protein. **a**, **b** and **c** represent an 0.1 μl aliquot applied 5, 3 and 2 times, respectively, to pure nitrocellulose with a Hamilton syringe. The nitrocellulose is thoroughly dried between each application. The stain is purple on a white background. The color developing reagent contains 5-bromo-4-chloro-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). Total concentration = 2 mg/ml; **a** = 0.5 μl; **b** = 0.3 μl; **c** = 0.2 μl.

Figure 2a (at left). Isoelectric focusing. Lanes 1 and 6 represent the purified protein which has an isoelectric point of 8.4; lanes 2 and 3 represent gallbladder stone extracts; 4 and 5 kidney stone extracts, and 7 is the standard on the far right with isoelectric point of 3 to 10. The samples are 5 μl; the standard 1 μl. The sample concentration is approximately 2 mg/ml; the standard concentration = 0.01 mg/μl.

Figure 2b (above). Isoelectric focusing. One μl of standard ph 3-10 on right. Five μl aliquots of purified protein on left (concentration 2 mg/ml). Arrow = 8.4.

Table 1. Percentage composition of amino acids of cationic protein.

Aspartic Acid/Aspargine	11.07
Threonine	13.37
Serine	4.34
Glutamic Acid/Glutamine	2.25
Isoleucine	3.73
Leucine	10.19
Tyrosine	2.58
Phenylanaline	12.80
Lysine	26.09
Histidine	6.04
Tryptophan	----
Arginine	7.53

and the values read on a spectrophotometer at 280 nm. Fraction II is pooled and dialyzed against large volumes of distilled water in the cold room, followed by concentration against PEG to a volume of 5-10 ml. The concentrated fraction is mixed with a small volume of SI-17 (Pharmacia LKB, Piscataway, NJ) in 0.09 M Tris-Glycine buffer, allowed to stand for at least 2 hours and centrifuged for removal of any pigment present. After ultracentrifugation, the protein containing peak is chromatographed by fast protein liquid chromatography (FPLC) on a polyanion SI-17 column (Pharmacia LKB). Solution A is tris-glycine and solution B is tris-glycine sodium chloride. The protein is eluted in the first peak which is concentrated and dialyzed against distilled deionized water. The purity is checked by immunodiffusion, SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

The molecular weight was estimated by SDS-PAGE (Figures 1a and 1b) and gel filtration (calibration kit; Pharmacia LKB); and the isoelectric point (Figure 2) was determined on agarose bound to a rigid support (FMC Bioproducts, Rockland, ME). Amino acid analysis (Table 1) and amino acid sequencing (see **Results**), immunodotting (Figure 3) and immunodiffusion were also performed. An Amplified Alkaline Phosphatase Goat Anti-Rabbit Immuno Assay Kit (Bio-Rad, Chemical Div., Richmond, CA) was used to assay the purified protein and an Immuno-Blot Alkaline Phosphatase (Goat Anti-Mouse, Bio-Rad) was employed to assay CD59 (monoclonal antibody, procured from Accurate Chemical and Scientific Corporation, Westbury, NY). The N-terminal amino acid sequence was performed on a peptide generated by digestion of the protein with cyanogen bromide and after separation of the peptides on a Vydac Reverse Phase C 18 column (4.6 mm x 25 cm) (the analysis was carried out by O/T Biotechnology Research Institute, Rockville, MD).

Results

Scanning with the antiserum to proteins of the urate-calcium oxalate stone revealed the presence of the protein in bile, cholesterol gallstones and extracts from the aorta. The molecular weight of approximately 82 kD on SDS PAGE is consistent with the result obtained on AcA44 gel filtration, and the isoelectric point of 8.4, classifying the protein as cationic, is compatible with the high content of basic amino acids.

Partial sequencing of a peptide obtained after cyanogen bromide cleavage of the protein necessitated by a blocked N-terminal revealed approximately 60% homology with CD59 (Protectin). Immunodotting and immunodiffusion with the CD59 monoclonal antibody failed to detect the purified protein.

The sequence of the peptide generated from the protein by cyanogen bromide cleavage follows:

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                    5
LEU-GLN-LEU-ASN-ASN-ARG
                   10
PRO-ASN-XXX-THR-ALA-ASP
                   15
GLU-LYS-THR-ALA
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Discussion

The constant identity of the two proteins, one extracted from stone and the other purified from urine, as displayed by electrophoretic mobility and immunological activity, suggests that the procedure of extraction preserves the native state of the protein. This would give more credence to the *in vitro* studies of the role of this and other similarly extracted proteins in the formation of urinary tract stones.

The cationic charge of the protein may be responsible for its presence in the stone through its linkage with the anionic proteins, and once combined, could form the elementary matrix ready to receive its first cargo of salts if such are available in a supersaturated solution.

The selective incorporation of the protein into stones and its distribution in calcification prone sites suggest a role for this protein in the process.

The partial homology with CD59 (Protectin) would enlarge the distribution and role of an isoform of this protein while the failure to react with a monoclonal antibody to CD59 leaves the new protein without identification. Further research on the nature and role of this interesting protein is being pursued.

Acknowledgements

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Discussion with Reviewers

M.I. Resnick: Do the authors believe that the protein is similar to proteins found in other stones such as pure calcium oxalate struvite? What role will proteins play in stones of this composition?

Authors: Yes, we believe that the protein is similar to proteins found in other stones such as pure calcium oxalate struvite. The role of the protein is speculative at the present time. It is being studied, *in vitro*, by physical chemical techniques.