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# Isoelectric focusing of Tamm-Horsfall glycoproteins: a simple tool for recognizing recurrent calcium oxalate renal stone formers

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**Abstract** Tamm-Horsfall glycoproteins (THPs) from healthy probands and a majority of recurrent calcium oxalate renal stone formers reveal different physicochemical properties when analyzed using isoelectric focusing (IEF). The pI values of THPs from healthy probands are approximately 3.5 while THPs from recurrent renal stone formers have pI values of between 4.5 and 6. The two groups of THPs exhibit completely different protein patterns. The differences in IEF analysis allow differentiation between THPs from healthy probands and recurrent calcium oxalate stone formers and may possibly be used as a simple diagnostic method for the recognition of recurrent calcium oxalate renal stone formers.

**Key words** Isoelectric focusing · Tamm-Horsfall glycoprotein · Renal stone formation · Differentiation · Diagnostic method · Recurrent calcium oxalate stone former

Urine of renal stone formers and healthy persons is nearly always supersaturated with respect to calcium oxalate, which favors nucleation and growth of these crystals [5]. The analysis of urinary electrolytes does not allow discrimination between calcium oxalate stone formers and healthy persons and is not predictive of an individual's risk of forming stones. The ion activity product of calcium oxalate in urine is at least 4 times higher than its solubility, meaning that crystal formation occurs in both groups, healthy persons and stone formers [3]. For that reason, supersaturation cannot be the sole explanation for calcium oxalate stone formation. The fact that several studies have identified naturally in urine excreted substances, e.g., proteins, with inhibitory effects on the precipitation of calcium oxalate [4, 20], rather suggests that the pathogenesis of calcium oxalate stone formation must be regarded as the result of an imbalance between supersaturation with calcium oxalate and urinary inhibitory activity [19].

Tamm-Horsfall glycoprotein (THP), a renal glycoprotein found in man and many other species, is one of the major glycoproteins excreted in human urine [23]. It is produced by the kidneys and has been localized in the thick ascending limb of the loop of Henle and the early distal, convoluted tubule segments of the nephron [16]. THP has a molecular weight of approximately 80 kDa; 25–30% of THP is accounted for by sialylated, sulfated and *N*-acetylgalactosamine (GalNAc)-containing *N*-linked carbohydrates [11].

THP seems to play an important role in renal stone formation. A functional diversity of THPs from recurrent calcium oxalate stone formers and healthy probands has been found. THPs from recurrent stone formers had no effect or promoting influences on the precipitation of calcium oxalate while healthy probands' THPs had inhibitory properties [13, 21]. Recent work has shown distinct structural differences in sialic acid content [15] and surface negative charge [1] between these two groups of THPs.

In the present study, the physicochemical properties of THPs from recurrent calcium oxalate renal stone formers and healthy probands were investigated using isoelectric focusing (IEF) in ultrathin gels, and a method was developed for a simple differentiation between these different THPs. This could be of diagnostic interest in the recognition of recurrent calcium oxalate stone formers.

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## Materials and methods

### Chemicals and reagents

Acrylamide, *N*,*N*'-methylene-bis-acrylamide, trichloroacetic acid (TCA), ammonium peroxodisulfate, Triton X-100, urea, glycine, TRIS, sulfuric acid and *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED) were obtained from Merck, Darmstadt, Germany. Sodium dodecyl sulfate (SDS), 2-mercaptoethanol and glycerol were obtained from Fluka, Buchs, Switzerland. Repel Silane was obtained from Pharmacia, Dübendorf, Switzerland. All chemicals used were at least of reagent grade. Carrier ampholytes used for IEF were of the "Servalyt" type from Serva, Heidelberg, Germany. The protein test mixture 3–10 for pI determination was obtained from Serva, Heidelberg, Germany, and consisted of cytochrome C (pI 10.65), ribonuclease A (pI 9.45), lectin (pI 7.75/8.0/8.3), myoglobin (pI 6.9/7.35), carbonic anhydrase (pI 6.0), β-lactoglobulin (pI 5.15/5.3), trypsin inhibitor (pI 4.5), glucose oxidase (pI 4.2) and amyloglucosidase (pI 3.5).

### Urine specimens of recurrent calcium oxalate stone formers and healthy probands

Urine specimens of stone formers were obtained from patients classified as recurrent calcium oxalate stone formers from the Kantonsspital Basel and Kantonsspital Baden. The patients were sex- and age-mixed and had no urinary tract infections. Urine specimens of healthy probands were obtained from randomly chosen healthy probands of both sexes and various ages, who had previously had no known stone episode and no other disease at that time.

### Protein isolation and sample preparation

Twenty-five THPs from healthy probands and 14 THPs from recurrent stone formers were isolated by a modified method of Tamm and Horsfall [22]. Urine samples (first morning urine) were precipitated 3 times with 0.58 mol/l NaCl and the precipitates were dialyzed against 0.05 mol/l TRIS-HCl buffer pH 7.8 for 72 h. The final products were lyophilized. For IEF sample preparation a known amount of THP was placed in an aliquot of sample buffer [9.5 mol/l urea; 2% (v/v) Triton X-100; 5% (v/v) 2-mercaptoethanol; 2% (w/v) Servalyt 3–10] and incubated for 20 min at 40 °C before use.

### IEF in ultrathin gels

Two glass plates (120 × 250 mm) with spacers (250 × 5 × 0.5 mm) were used to cast polyacrylamide gels using the technique of Görg et al. [9]. An acrylate-activated polyester sheet [Gel-Fix for polyacrylamide gel electrophoresis (PAGE), Serva, Heidelberg, Germany] was fastened to the upper plate by capillary attraction, and the lower plate with molds for gel slots was pretreated with Repel Silane. The gel solution [16.65% (v/v) acrylamide/*N*,*N*'-methylene-bis-acrylamide (28.8/1.2); 26.7% (v/v) 38% glycerol; 5% (v/v) Servalyt 3–5 (gradient pH 3–5); 1.65% (v/v) Servalyt 3–10; 50% (v/v) 4 mol/l urea solution] was degassed in vacuo for 3 min before use. Polymerization of the gel solutions was catalyzed by 15 µl TEMED and 25 µl of a 40% (w/v) ammonium peroxodisulfate solution. Polymerization took place at 50 °C for 60 min. IEF was performed at 4 °C using a Multiphor II electrophoresis unit (Pharmacia, Dübendorf, Switzerland). Sulfuric acid 0.05 mol/l and a 2% (w/v) glycine solution were used as analyte and catholyte, respectively. After a 150-V h prerun at 10 mA constant current, 15-µl samples (protein concentration 1 mg/ml) were loaded in the gel slots. Focusing conditions were set to 50 V h at 200 V constant voltage and then to 3500 V h at 2500 V constant voltage ($I_{max}$ = 10 mA; $P_{max}$ = 15 W). The pH gradient was determined by pI-marker proteins.

### Staining

After fixing with a 20% (w/v) TCA solution in *n*-isopropanol/water (1/1, v/v) for 60 min the gels were stained with a 0.1% (w/v) Coomassie Brilliant Blue G-250 solution in methanol/water/acetic acid (5/4/1, v/v/v) for 60 min and destained with destainer (methanol/water/acetic acid, 9/10/1, v/v/v) until the background was clear.

### Densitometric scanning

Stained gels were scanned with a CAMAG scanner TLC-2 (CAMAG AG, Muttenz, Switzerland). The densitometer curves were processed with CAMAG CATS II V3.14 software.

## Results

Due to the urea and detergent content of the sample buffer and the necessary urea content of the gels used, an accurate assignment of the absolute pI values of the glycoproteins is difficult [7]. Nevertheless, IEF of 25 THPs from healthy probands in ultrathin gels with carrier ampholytes pH 3–5 showed a diffuse, barely focused double lane at approximately pI 3.5 (Fig. 1). Conversely, 10 of 14 THPs from recurrent calcium oxalate stone formers showed sharply focused multiple lanes with pI values in the range from 4.5 to 6, exhibiting approximately the same basic pattern of focused proteins (Fig. 2). However, 4 of 14 THPs from recurrent stone formers focused in the same way as the THPs from healthy probands. Densitometric scans of the IEF patterns of THPs from a healthy proband and a recurrent calcium oxalate stone former are shown in Fig. 3.

## Discussion

Application of IEF in ultrathin gels revealed distinctly different pI values and protein patterns of THPs from healthy probands and a majority of recurrent calcium oxalate stone formers. THPs from healthy probands showed two slurred bands with an approximate pI 3.5 in good agreement with earlier results [17], while most of the THPs of recurrent stone formers focused sharply with a multiple-lane protein pattern and with pI values in the range from 4.5 to 6. The apparent difference in pI value may have several implications for the role of THP in renal stone formation and can explain the functional diversity of THPs from recurrent stone formers and healthy probands. The low pI value of THPs from healthy probands shows that it is an acidic macromolecule. According to the proposed mechanism by which urinary macromolecules inhibit crystal growth and aggregation [18], such an anionic molecule could bind to the surfaces of growing calcium oxalate crystals, block the growing sites and modify attractive or repulsive forces between the crystals, thereby impeding or preventing aggregation of the crystals. Consequently [6], no large crystal clusters would be formed to obstruct the renal tubules and any microcrystals

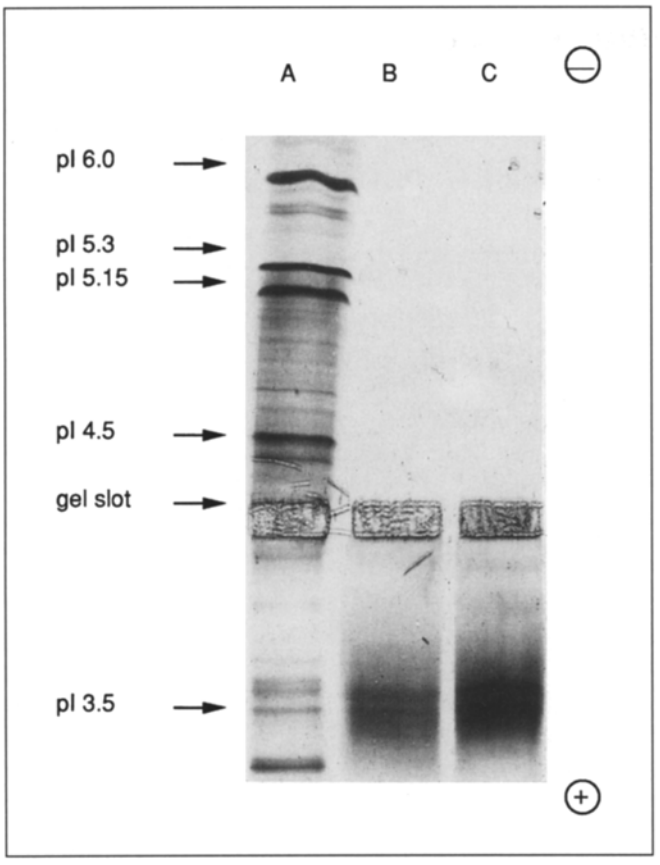


**Fig. 1** Characteristic IEF patterns of THPs from healthy probands in ultrathin gels with carrier ampholytes, pH 3–5. *A* IEF marker proteins, *B*, *C* THP of healthy probands

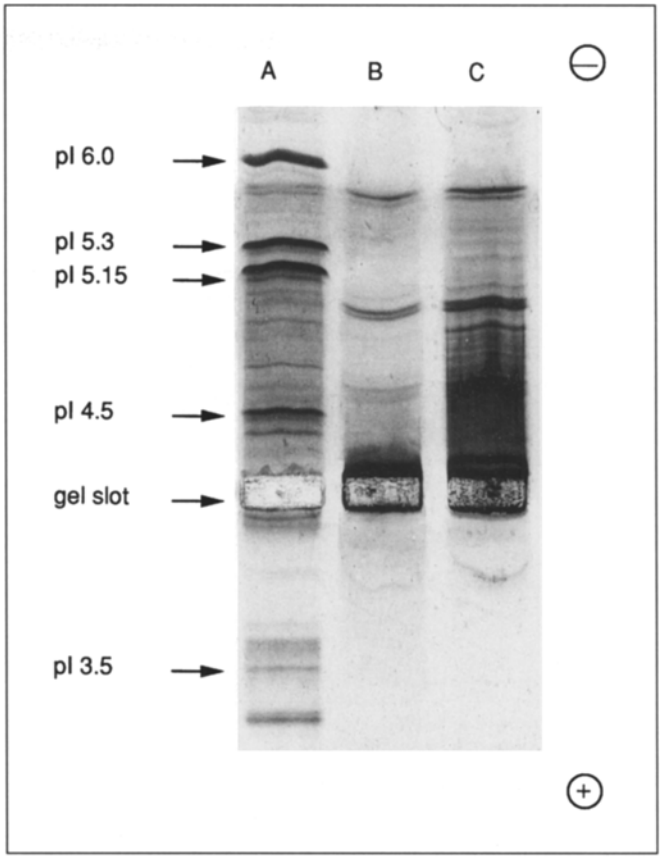


**Fig. 2** Characteristic IEF patterns of THPs from recurrent calcium oxalate renal stone formers in ultrathin gels with carrier ampholytes, pH 3–5. *A* IEF marker proteins, *B*, *C* THP of recurrent stone formers

formed due to supersaturation could easily be washed out from the urinary tract. THPs having more basic pI values, which must correspond to a smaller content of negatively charged groups, as is found in patients with calcium oxalate urolithiasis, would no longer be capable of binding effectively to these calcium oxalate crystals. In contrast, these THPs present an uncharged surface that might act as an additional surface for heterogeneous nucleation and thus provide a framework for the deposition of stone-forming salts as proposed earlier [2]. This result supports the idea that stone formers are no longer fully protected against the formation of large crystal aggregates, which can be deposited in the urinary tract [12].

The reason why the two functionally different THPs from healthy probands and recurrent calcium oxalate stone formers have different physicochemical properties can be explained by an altered glycoprotein structure resulting from a different chemical composition of these THPs. The different sialic acid contents of these two groups of THPs may at least be one of the causative factors but the differences in the pI values could also be influenced by different sulfate-group contents or by different contents of carboxy-groups from amino acids. However, the reason that there are some THPs from recurrent stone formers that migrate like THPs from healthy probands may be that renal stone formation is a complex, multifactorial disease with several causes of recurrent stone formation [8]. The altered glycoprotein structure of THP, like the lack of sialylation or sulfation, is reminiscent of the carbohydrate-deficient glycoprotein (CDG) syndrome, an inherited metabolic disorder affecting glycoprotein metabolism [10]. In this case the serum glycoprotein transferrin shows a partial sialic acid deficiency and IEF of serum transferrin is used as a reliable diagnostic test in the diagnosis of the CDG syndrome [14].

Since there is no reliable parameter for the prediction of recurrence in renal stone-forming patients and because of the important role THP seems to play in the recurrent stone-forming process, a knowledge of the type of THP a patient excretes (acidic healthy probands' THP or more basic recurrent stone formers' THP) would be of great diagnostic interest in the classification of renal stone-forming patients. Until now only a limited number of THPs from recurrent stone formers and healthy probands have been analyzed, but IEF analysis of patients' THP could possibly be able to help in the prediction of a patients' risk of forming recurrent renal stones because IEF can differentiate very easily

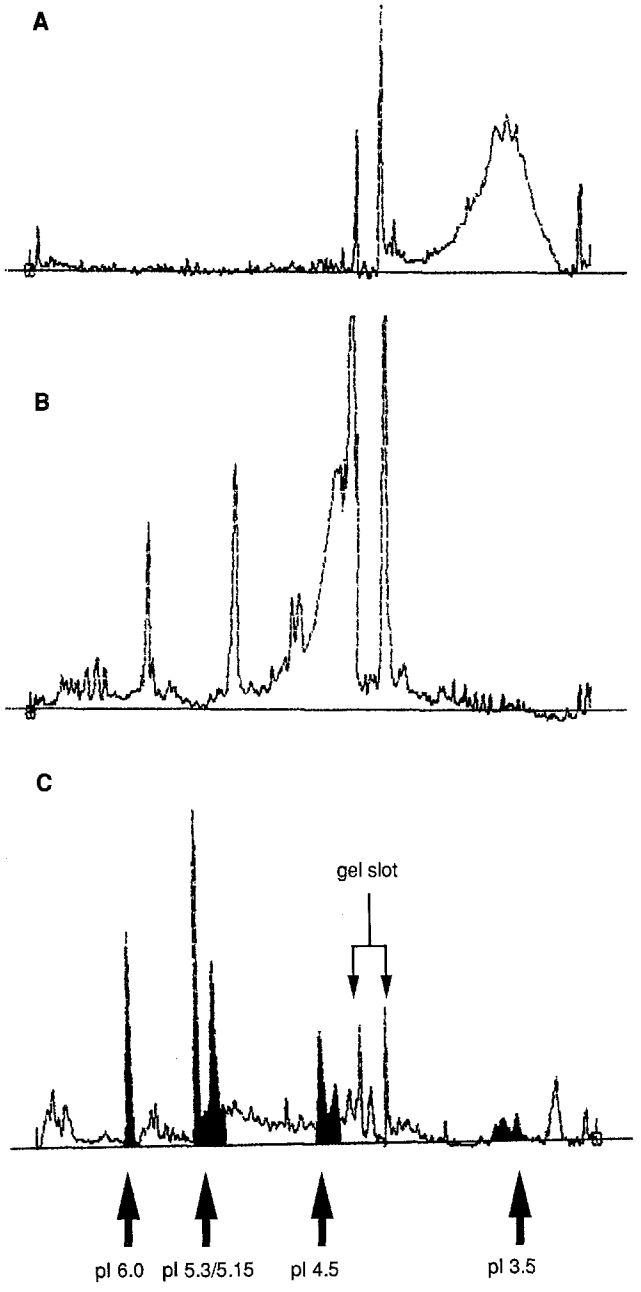


**Fig. 3** Densitometric profiles of IEF patterns of THPs. **A** THP from a healthy proband, **B** THP from a recurrent calcium oxalate stone former, **C** IEF marker proteins

between these two groups of functionally and structurally different THPs.

In conclusion, IEF reveals different physicochemical properties of THPs from healthy probands and most of the recurrent calcium oxalate renal stone formers. The two groups of THPs exhibit completely different protein patterns. These differences in IEF analysis allow differentiation between THPs from healthy probands and recurrent calcium oxalate stone formers and may possibly be used as a simple diagnostic method in the recognition of recurrent calcium oxalate stone formers.

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