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A simple diagnostic method for the differentiation of Tamm-Horsfall glycoproteins from healthy probands and those from recurrent calcium oxalate renal stone formers

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Abstract. Tamm-Horsfall glycoproteins (THPs)* from healthy probands, and those from a majority of recurrent calcium oxalate renal stone formers, reveal different properties when analyzed using isoelectric focusing. The pI-values of THPs from healthy probands are approximately 3.5 while THPs from recurrent renal stone formers have pI-values between 4.5 and 6. The two groups of THPs exhibit completely different protein patterns in IEF. This proves the structural difference of these THPs. The differences in IEF analysis allow the differentiation between THPs from healthy probands and those from recurrent calcium oxalate stone formers. These differences could 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.

Tamm-Horsfall glycoprotein (THP) is a renal glycoprotein found in man and many other species. The glycoprotein 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[1]. It is one of the major glycoproteins excreted in human urine[2]. THP has a molecular weight of approximately 80 kD; 25–30% of THP is accounted for by sialylated, sulfated and GalNAc containing N-linked carbohydrates[3]. THP has been studied extensively, but its physiological function is still controversial[4]. There is evidence that THP is implicated in certain pathological states[5,6]. Its role in renal stone formation is most prominent[7]. 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, the most common component of renal stones, whereas THPs from healthy probands had inhibitory effects[8,9]. Recent work showed distinct structural differences in sialic acid content[10] and surface negative charge[11] between these two groups of THPs.

In the present study, isoelectric focusing in ultrathin gels was used to further characterize the specific properties of THPs from recurrent calcium oxalate renal stone formers and healthy probands, and methods were developed for a simple differentiation between these different THPs. These could be of diagnostic interest in the recognition of recurrent stone formers.

*You will find a list of the abbreviations used at the end of the text.

Materials and methods

Chemicals and reagents. Acrylamide, N,N′-methylene-bis-acrylamide, TCA, ammonium peroxodisulfate, Triton X-100, urea, glycine, Tris, sulfuric acid and TEMED were from Merck (Darmstadt, Germany). SDS, 2-mercaptoethanol and glycerol were from Fluka (Buchs, Switzerland). Repel Silane was from Pharmacia (Dübendorf, Switzerland). All chemicals used were at least of reagent grade. Molecular weight markers for SDS-PAGE were from Biorad (Glattbrugg, Switzerland). Carrier ampholytes used for IEF were of 'Servalyt' type from Serva (Heidelberg, Germany). Protein test mixture 3–10 for pI determination was 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), glucoseoxidase (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 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 from randomly chosen healthy probands of both sexes and various ages, who had had no known stone episode so far and had no other disease at that time.

Protein isolation and sample preparation. 14 THPs from healthy probands and 14 THPs from recurrent stone formers were isolated by modification of the method of Tamm and Horsfall[12]. Urine samples (first morning

urine) were precipitated three 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. The purity of the isolated proteins was checked by SDS-PAGE[13]. For IEF sample preparation a known amount of THP was added to 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 by the technique of Görg et al.[14]. An acrylate activated polyester sheet (Gel-Fix for 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 three minutes 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 during 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 Vh prerun at 10 mA constant current, 15 µl samples (protein concentration 1 mg/ml) were loaded into the gel slots. Focusing conditions were set to 50 Vh at 200 V constant voltage and then to 3500 Vh 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) till the background was clear.

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

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[15]. Nevertheless, isoelectric fo-

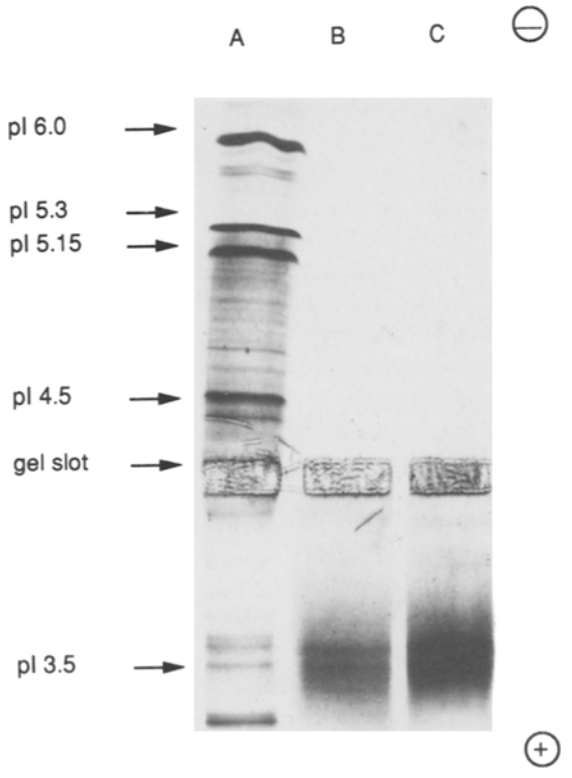

Figure 1. Characteristic IEF patterns of THPs from healthy probands in ultrathin gels with carrier ampholytes pH 3–5. *A*) IEF marker proteins; *B,C*) THP of healthy probands.

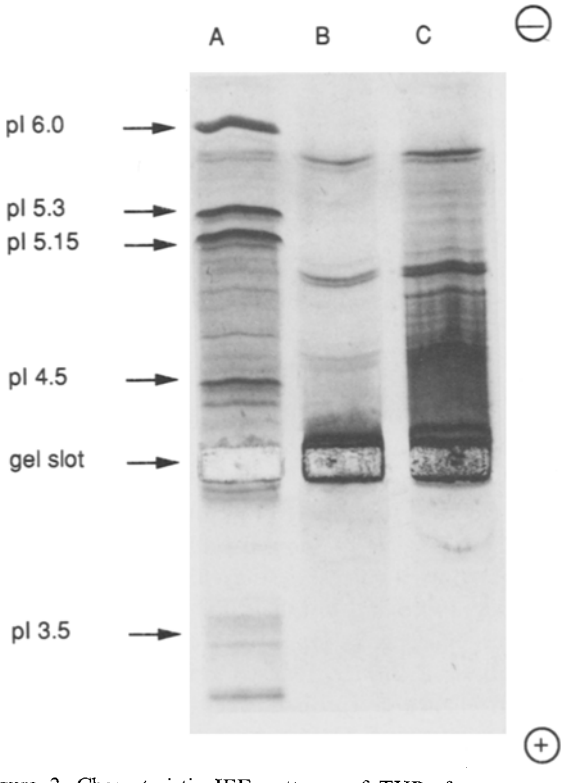

Figure 2. Characteristic IEF patterns of THPs from recurrent calcium oxalate renal stone formers in ultrathin gels with carrier ampholytes pH 3–5. *A*) IEF marker proteins; *B,C*) THP of recurrent stone formers.

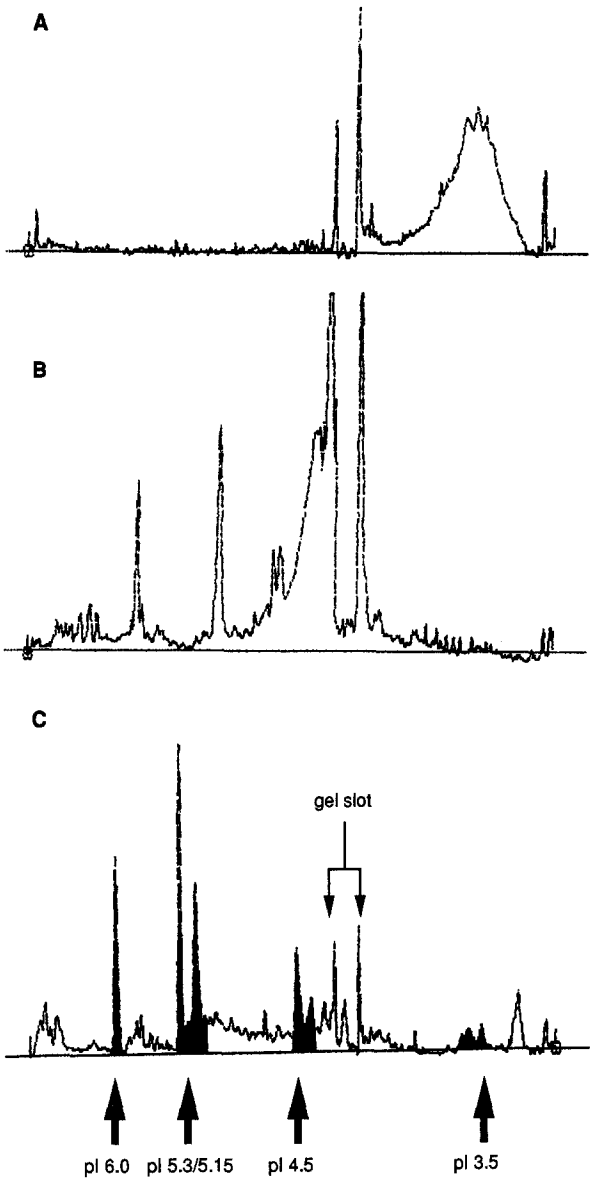

Figure 3. Densitometric profils of IEF patterns of THPs. *A*) THP from a healthy proband; *B*) THP from a recurrent calcium oxalate stone former; *C*) IEF marker proteins.

cusing of 14 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 multiple lanes focusing sharply, with pI-values in the range from 4.5 to 6, exhibiting approximately the same basic pattern of focused proteins (fig. 2). However, 4 out 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 calcuim oxalate stone former are shown in fig. 3. SDS-PAGE analysis of THPs from healthy probands and recurrent calcium oxalate stone formers always showed one lane with no other protein impurities migrating with a molecular weight of approximately 80000 D (fig. 4). Only slight differences in molecular weight, if any, could be seen between samples from recurrent stone formers and from healthy probands.

Discussion

Application of isoelectric focusing 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-value of 3.5, in good agreement with earlier results[16], while most of the THPs of recurrent stone formers focused sharply with a multiple lane protein pattern of 1 to 2.5 pI-values more basic. The apparent difference in pI-value may have 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 THP from healthy probands shows that it is an acidic macromolecule. According to the proposed mechanism by which urinary macromolecules inhibit crystal growth and aggregation[17], such an anionic molecule could bind to the surface of growing calcium oxalate crystals, block the growing sites, and modify attractive or repulsive forces between the cyrstals, thereby impeding or preventing aggregation of the crystals. Consequently[18], no large crystal clusters would be formed to obstruct the renal tubules and any microcrystals formed due to supersaturation could easily be washed out from the urinary tract. THPs from recurrent calcium oxalate stone formers, having more basic pI-values which must correspond to a smaller content of negatively charged groups in these glycoproteins, would no longer be capable of binding effectively to these calcium oxalate crystals. In contrast, these THPs would 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[19]. 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[20].

The fact that the two functionally different THPs from healthy probands and from recurrent calcium oxalate stone formers have different physicochemical properties can be explained by differences in the glycoprotein structure resulting from a different chemical composition of these THPs. The different sialic acid contents of the two groups of THPs may 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. There are some THPs from recurrent stone formers that

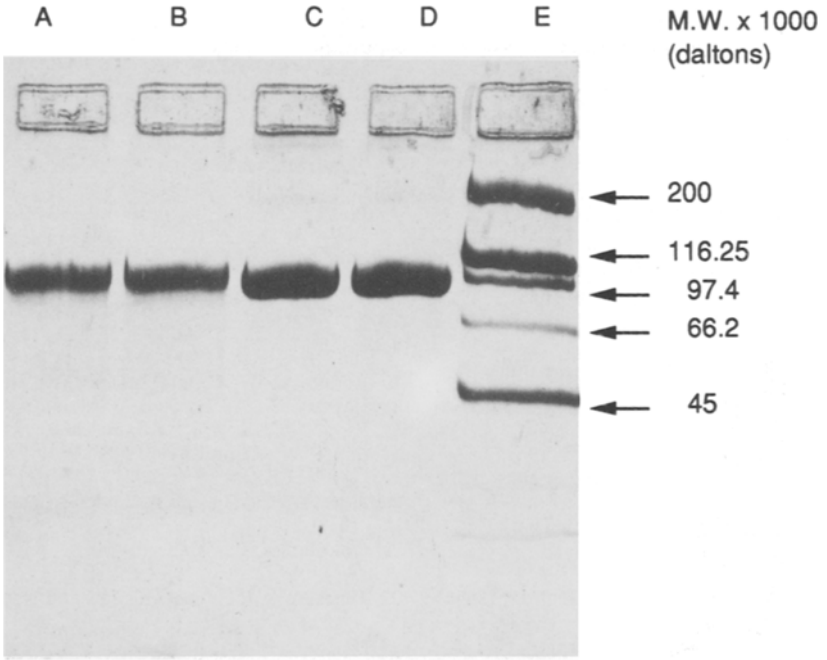

Figure 4. SDS-PAGE (gradient gel: 4–22.5%) of THPs from healthy probands and recurrent calcium oxalate stone formers. Running conditions: 100 min, 600 V (max), 50 mA (max), 30 W (max), 5 °C. Coomassie Brilliant Blue G-250 staining. *A,B*) THP of healthy probands; *C,D*) THP of recurrent calcium oxalate stone formers; *E*) SDS-PAGE marker proteins.

migrate like THPs from healthy probands. This indicates that renal stone formation is a complex, multifactorial disease, and there may be several causes for recurrent stone formation[21]. Using SDS-PAGE a molecular weight of approximately 80000 D and a high purity of the isolated proteins was revealed, but no difference in purity of the samples could be detected between THPs from recurrent stone formers and healthy probands, which is consistent with other investigations[22]. At most, slight differences in molecular weight between the different samples could be seen. This means that any differences in glycoprotein structure must be such that they have no observable effect on the migration in SDS-PAGE. The varying sialic acid contents of the two different groups of THPs, or even the entire lack of an N-glycan would be examples for such alterations.

An altered glycoprotein structure of THP, like the lack of sialylation or sulfation, recalls the carbohydrate deficient glycoprotein (CDG) syndrome, an inherited metabolic disorder affecting glycoprotein metabolism[23]. In this case, the serum glycoprotein transferrin shows a partial sialic acid deficiency, and isoelectric focusing of serum transferrin is used as a reliable diagnostic test in the diagnosis of the CDG syndrome[24].

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 the more basic recurrent stone formers' THP) would be of great diagnostic interest in the classification of renal stone forming patients. Although until now only a limited number of THPs from recurrent stone formers and healthy probands have been analyzed, the results indicate that IEF analysis of patients' THP could possibly help in the prediction of a patients' risk of forming recurrent renal stones, because IEF can differentiate very easily between these two groups of functionally and structurally different THPs.

In conclusion, isoelectric focusing reveals different physicochemical properties of THPs from healthy probands and most of the recurrent calcium oxalate renal stone formers. This proves that they are structurally different. These differences in IEF analysis allow differentiation between THPs from healthy probands and those from recurrent calcium oxalate stone formers, and could possibly be used as a simple diagnostic method in the recognition of recurrent calcium oxalate stone formers.

Abbreviations

THP	Tamm-Horsfall glycoprotein
IEF	Isoelectric focusing
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
kD	Kilo Dalton
D	Dalton
CBB G-250	Coomassie Brilliant Blue G-250

Tris	Tris(hydroxymethyl)aminomethane
GalNAc	N-acetylgalactosamine
TEMED	N,N,N′,N′-tetramethylethylenediamine
M.W.	Molecular weight
P	Power
I	Current
CDG	Carbohydrate deficient glycoprotein

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