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Research Article

Production and confirmation of polyclonal antibody against tamm horsfall protein and its application in kidney stone disease diagnosis

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ABSTRACT

Increasing numbers of the kidney stone cases, all over the world, urgently demands simple and reliable method of detection, in which measuring the expression of biomarker level has already gained considerable success. Tamm Horsfall Protein (THP), biomarker for kidney stone, acts as the major inhibitors of stone formation (crystal growth) in normal urine but due to its abnormality its inhibitory effect is lost and growth of stone is promoted. Herein, we have isolated THP by four different methods namely Sodium chloride, Ammonium sulphate, Acetone, Polyethylene glycol and purified from human urine samples by column chromatography which were confirmed by SDS PAGE band at 97 kDa. PAGE analysis revealed that four protein bands were seen in Ammonium sulphate followed by sodium chloride method. Also decreased excretion of THP was found in case of stone patients and also aggregation of THP was more in stone patients than compared to normal which was established by SEM. Polyclonal antibodies against purified THP protein (antigen) was produced and confirmed. Our results indicate that this is the easy way to screen and differentiate the normal subjects from the renal stone patients excreting abnormal THP levels. Thus, the present method holds huge promises to design a diagnostic kit for detection of kidney stone in future.

Keywords: Kidney stone diseases; Tamm Horsfall Protein; SDS-PAGE; Polyclonal antibody.

INTRODUCTION

Kidney stone is a common problem of society in present times as it affects 1 – 20% of the general population which varies in relation to age, race and gender. Kidney stone disease afflicts both men and women but with higher prevalence in men than in women and the risk of reoccurrence is 74% within 10 years for the first time stone formers. Formation of stone in kidney is a complex and multifactorial process taking place in supersaturated urine, where the urine substances form crystals that stick together and subsequently grow into stones on the inner surface of the kidney (Hess B and-Kok DJ, 1996). Calcium stones are the most predominant that composes 75 – 80% of all stones (Fredric L. Coe*et al*., 2005) and its reoccurrence is greater than other types of kidney stones. In human, the mechanism against the formation of kidney stone involves macromolecules, i.e., protein and glycosaminoglycan which are polyanionic molecules with substantial amounts of acidic amino acid residues that inhibit crystal aggregation (Robertson WG*et al*., 1976).

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Tamm Horsfall Protein (THP) is the most abundant protein (85 kDa) in the normal human urine (Kumar S *et al*., 1990) secreted by the thick ascending limb of the loop of Henle (Gokhale J. A*et al*., 1996). It is implicated as both an inhibitor and promoter of stone formation (Hess B*et al*., 1989). Its inhibitory properties arose by coating crystals, retarding the attachment of new crystals and thus preventing crystals growth and aggregation. However, self-aggregation and polymerisation of THP is caused by high calcium concentration, high ionic strength and low pH (Steveson F.K. and Kent P. W 1970), which allows the protein to act as a promoter by forming a mesh to which crystals adhere and thus initiate crystal growth. Hess *et al*. (1989) described that THP is the major inhibitor of calcium oxalate crystal aggregationin the urine of healthy subjects. However, stone formers excreted defective urinary THP that diminishes its inhibitory effect on stone formation (Hess B*et al*., 1989). Low pH, high ionic strength and high concentrations of calcium and THP itself all favour self-aggregation of THP molecules which lowers their inhibitory activity against calcium oxalate crystal aggregation (Hess B, 1994). There is a decreased excretion of THP in stone formers (Lau W.H*et al*., 2008). The range of excretion of TH Glycoprotein per ml of creatinine clearance was greater than in normal subjects, independant of the type of renal disease and unrelated to proteinuria.

In patients with glomerulonephritis the excretion of Tamm-Horsfall glycoprotein per ml of creatinine clear-

ance was significantly higher in those with well - preserved tubules compared with those with tubular atrophy. In the presence of calcium, increasing THP concentrations progressively lowered inhibition by THP of stone former, but not by normal THP (Hess B*et al*., 1993). However it is described that recurrent calcium stone formers with a positive family history excrete more THP than healthy controls and increasingly aggregated THP molecules predispose to exaggerated calcium oxalate crystal aggregation, an important prerequisite for urinary stone formation (Jaggi M*et al*., 2007). The contradictory data obtained by these various studies may be due to the different proportion of free and aggregated forms of THP in patients' urine; furthermore, the two THP forms have different solubility in salts solutions (Kobayashi K and Fukuoka S, 2001).

By these observations, it has been suggested that not only defective THP but an abnormal levels of THP excretion may facilitate the development of kidney stones. In the stones, the THP from the healthy subjects was more glycosylated with sialic acid than the recurrent stone formers (Knorle R et al., 1994). The terminal sialic acid is essential for the inhibitor function of THP in healthy subjects; however this function was diminished in the recurrent stone formers owing to their THP lack of sialic acid. Thereafter, carbohydrate structure of THP is the major feature that may regulate kidney stone formation. From the above discussion it has been postulated that urinary THP may be useful marker for renal stone formation. In the present study, precipitation of THP was done by four different methods; purification and quantification of THP from the samples were also done. Further the purified THP was injected into the mice models and confirmation of polyclonal antibody formation was done by Ouchterlony double diffusion method (ODD).

MATERIALS AND METHODS

Materials

The urine samples were granted by the Madras Medical Mission hospital (Chennai). Sodium chloride, sodium dihydrogen phosphate, acetone and disodium hydrogen phosphate were the products of Merck (Mumbai, India). Ethylenediaminetetraaceticacid, ammonium sulphate, tween 20 and sodium azide were purchased from Alfa Aesar (Hyderabed, India). Concanavalin A Sepharose was obtained from Sigma Aldrich (Bangalore, India). Calcium chloride, silica gel, sodium hydroxide and sodium carbonate were purchased from LobaChemiePvt. Ltd., (Mumbai, India). Copper sulphate, sodium potassium tartarate, folin's reagent, acrylamide and coomassie brilliant blue were obtained from Thomas Baker Chemicals (Mumbai, India).

Collection of urine samples

Table 1 gives the result of ultrasound study and the calculus measurement. Urine samples were collected from healthy and stone forming individuals of the Madras Medical Mission hospital (Chennai). Stone patients had been confirmed by radiography using plain abdominal radiography. Creatinine concentration in urine samples was measured and the assay was relied on the Jaffe's reaction.

Isolation and extraction of THP protein

The normal urinary proteins were isolated by precipitation. The four different precipitating agents were used which includes sodium chloride (Carvalho M. R*et al*., 2002), acetone, ammonium sulphate and polyethylene glycol (Raga Sudha M*et al*., 2012).

Isolation of THP Protein

Firstly, the sodium chloride method was used to precipitate the protein (Kumar S*et al*., 1990). Briefly, the solid Sodium chloride (0.58 M) was added to the urine sample (50 ml) and stirred overnight. After the centrifugation, the precipitate was washed with aqueous solution (0.58 M) and is dissolved in one-tenth volume of water and is dialysed against a litre of water. Secondly, equal quantities (7 ml each) of urine and cold acetone were mixed thoroughly and centrifuged at 2500 g for 30 mins at 4 °C. The precipitate was air dried and was carefully dissolved in phosphate buffer saline (200 ml).

Thirdly, two separate methods were used. In one of the methods, Ammonium sulphate (32 gm) was dissolved in the urine sample (50 ml) and stirred for 24 hrs. Centrifugation was done at 2500 g for 30 mins at 4°C and the sediments were dissolved in Phosphate buffer saline (100 ml). In the other method, the urinary proteins were extracted from 1 ml of urine sample by ammonium sulphate salt precipitation. To the aliquot urine (1 ml) added to ammonium sulphate (0.27 gm) was added and vortexed gently for 2 mins. After centrifugation, the pellet was dissolved in Phosphate buffer saline. Finally, 3 ml of PEG (4%) was added to the urine sample (30 ml) and centrifuged at 10000 rpm for 30 mins at 4 °C; 40% PEG was added to the pellet and again centrifuged. The supernatant was collected and stored.

Purification of THP protein

THP protein was purified using the chromatographic technique with the Concavalin A Sepharose column. The samples were passed through the column and eluted. All the samples after running through the column were desiccated using calcium chloride, silica gel and sodium hydroxide as desiccants.

Creatinine and Protein assay

The fresh and precipitated urine samples were used to estimate the Creatinine concentration. It was performed by the Jaffe's reaction method, in which the red orange colour was formed by the reaction of alkaline picrate with creatinine. Urinary Creatinine concentration was used as the correction factor for the effect of urine hydration on THP concentration. The protein was estimated using Lowry's method.

Table 1: Ultra sound results of patients

S.No	Patient Details			Ultra Sound Result	Calculus
	Name	Age	Gender		Measure (mm)
1	Baskar	46	M	Left hydroureteronephrosis	9
2	SadhiqBasha	39	M	No abnormalities	-
3	Ramesh Kumar	41	M	Right hydroureteronephrosis	7
4	Venketeswaran	49	M	Right hydroureteronephrosis	9
5	Gowtham	40	M	Left hydroureteronephrosis	5
6	Lokesh	40	M	Left hydroureteronephrosis	7
7	Yaswanth	40	M	No abnormalities	-
8	Dinakaran	44	M	Right hydroureteronephrosis	8
9	Moorthy	42	M	No abnormalities	-
10	Prakash	40	M	Right hydroureteronephrosis	5
11	Muthulingam	38	M	Left hydroureteronephrosis	9
12	Pavan Kumar	43	M	Right hydroureteronephrosis	9
13	Ravi Kumar	44	M	Left hydroureteronephrosis	8
14	Solomon	44	M	No abnormalities	-
15	Balaji	40	M	Right hydroureteronephrosis	6
16	Alice	48	F	No abnormalities	-
17	Rajalakshmi	42	F	No abnormalities	-
18	Malar	40	F	Left hydroureteronephrosis	8
19	Rathna	41	F	No abnormalities	-
20	Prabhavathy	40	F	No abnormalities	-

Table 2: Comparision of protein concentration and Creatinine in precipitated and fresh urine sample

Precipitating Method	Protein (PC)(g/ml)	Creatinine (CC) (mg/ml)	PC/CC (g/mg)
Sodium chloride	0.173	0.7	0.252
Ammonium sulphate 1	0.111	0.7	0.158
Ammonium sulphate 2	0.177	0.7	0.247
Acetone	0.032	0.7	0.045
Polyethylene Glycol	0.024	0.7	0.034
Fresh urine normal	0.190	0.7	0.271
Fresh urine patient	0.220	0.7	0.314

Figure 1: SDS-PAGE of protein precipitates of urine samples by Silver nitrate staining

(L1: molecular weight marker, L2: PEG precipitated sample, L3: Acetone precipitated,L4: Sodium chloride precipitated sample, L5: Ammonium sulphate1, L6: Ammonium sulphate 2 precipitated samples L7: molecular weight marker)

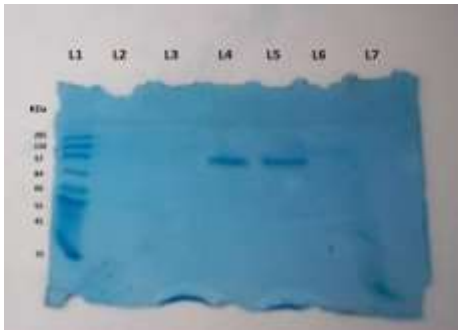

Figure 2: SDS PAGE of protein precipitates of urine samples by Coomassie brilliant blue staining

(L1: molecular weight marker, L2: PEG precipitated sample, L3: Acetone precipitated, L4: Sodium chloride precipitated sample, L5: Ammonium sulphate 2, L6: Ammonium sulphate 1 precipitated samples L7:molecular weight marker)

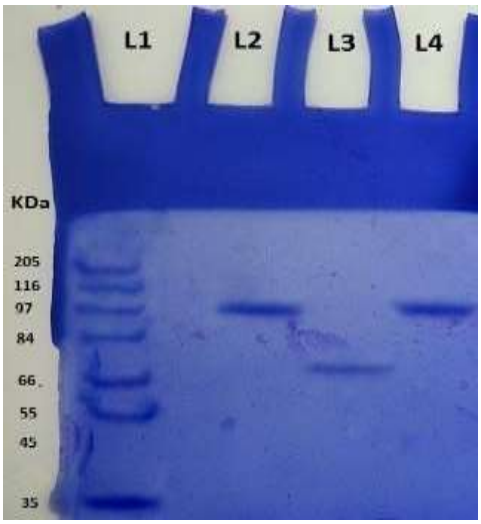

Figure 3: SDS PAGE of protein precipitates of urine samples by Coomassie brilliant blue staining

(L1: Molecular weight marker, L2: Sodium chloride, L3: Standard BSA, L4: Ammonium sulphate 2)

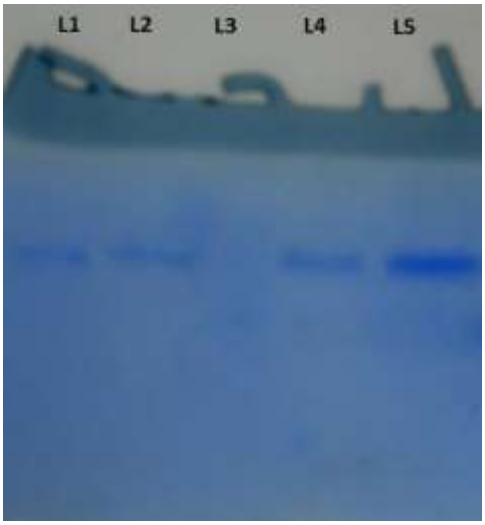

Figure 4: SDS PAGE of protein precipitates of patient and normal urine samples by Coomassiebrilliant blue staining

(L1: Stone patient sample A, L2: Stone patient sample B, L3: Stone patient sample C,L4: Stone patient sample D, L5: Normal sample)

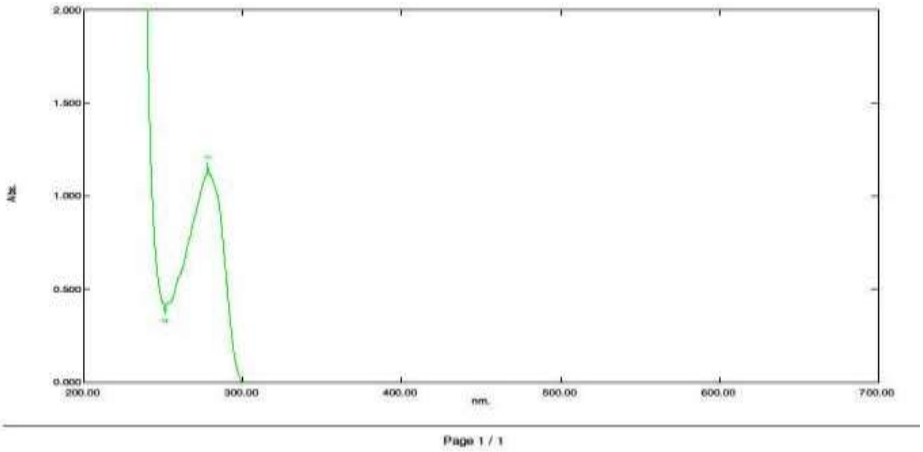

Figure 5: UV Spectrometry of normal THP

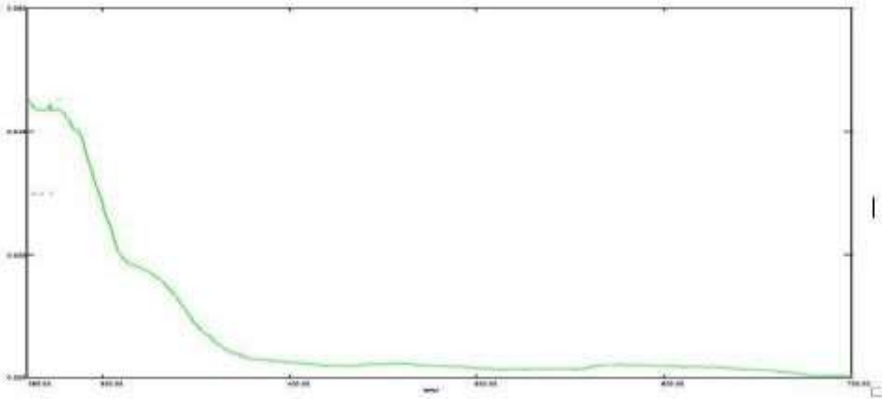

Figure 6: UV Spectrometry of disease THP

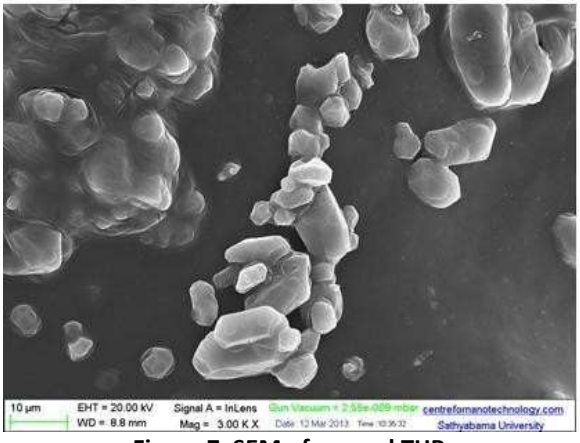

Figure 7: SEM of normal THP

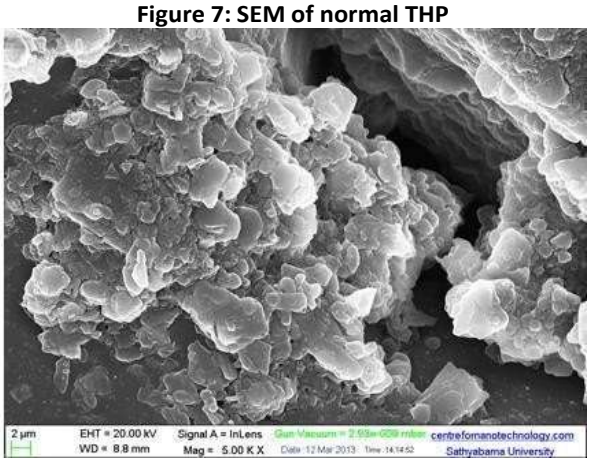

Figure 8: SEM of disease THP

Figure 9: Ouchterlony double diffusion pattern of polyclonal antibody against THP

Quantification and analysis of THP Protein

The isolated THP protein was quantified using the SDS-PAGE. The protein molecular marker was run along with the samples in order to quantify the THP protein. Silver staining was also performed for cross checking the results obtained. The THP protein concentration

was determined by the UV spectrophotometric analysis. The characteristics of the normal THP and diseased THP were analysed using the Scanning Electron Microscopy.

Production and confirmation of Polyclonal antibody

The polyclonal antibody production was carried out in Tamilnadu veterinary and animal sciences universi ty (Chennai). Preimmunized blood was collected from Balb/C mice model. The blood was centrifuged at 10000 rpm for 15 mins and the supernatant was stored which served as the control serum. The initial antigen (purified THP) injection (0^{th} day) was given in subcutaneous which includes Freund's Complete Adjuvant (FCA). First booster injection was given on 7^{th} day subcutaneous with Freund's Complete Adjuvant and the second booster injection was given subcutaneous on 14^{th} day. The third booster was given intravenous in the tail vein of the mice. After two days the blood was collected and the antibody production was confirmed by the Ouchterlony double diffusion method.

RESULTS AND DISCUSSIONS

The protein separation can be done by several methods like salt precipitation method, using organic solvent, and non-ionic polymers. In the present study, THP in urine was separated by four different methods namely by using Sodium chloride, Polyethylene glycol, Acetone andAmmonium sulphate. Precipitation of urinary protein was found to be efficient using ammonium sulphate and sodium chloride as most of the proteins precipitates in the presenceof high salt concentration; while organic solvents with small dielectric constants like acetonediscourages the dispersion of protein molecules in the media. Thus, the solubility of proteins can be lowered and precipitation can be induced. The use of non-ionic polymers such as Polyethylene glycol (PEG) for the precipitation is a method that can help prevent protein denaturation as well as the formation and equilibration of precipitates take significantly less time with PEG.

Protein quantification

Figure 1 and 2 shows the SDS-PAGE of protein precipitates of urine samples by Silver nitrate staining and Coomassie brilliant blue staining respecti vely. The SDS-PAGE analysis was performed for both healthy and stone patients and a decrease was observed in stone samples in comparison to the control of THP protein which was found as 97 kDa. The comparison between the THP protein between normal and stone patients is shown in Figure 3 and 4. The level of THP excretion and its correlation to citrate in urine are potent factors in the kidney stone forming process. The stone formers showed decreased THP excretion and described that the role of THP is to inhibit stone formation, normal person has higher THP levels as a protective measure against stone formation.

Table 2 shows the comparison of protein concentration and creatinine in precipitated andfresh urine samples. The protein concentration in precipitated samples of sodium chloride, ammonium sulphate were higher compared to other samples which commensurate with the results of SDS PAGE. Also protein content of stone patients was higher than the normal subjects. The PC/CC ratios in stone patients were higher than normal. The PC/CC ratio 0.2 is considered as normal but a higher ratio is an indication of renal disease or tubular dysfunction.

UV Spectrometric Analysis

UV Spectrometric analysis was performed for both normal and disease THP protein (Fig. 5 and 6). The maximum absorbance was observed at 278 nm and at 272 nm for the normal and disease THP respectively. The shift in the peak indicates the possible structural abnormalityor defective THP in stone patients. The defective THP may result in formation of stones (Hess B*et al.*, 1989).THP from the healthy subjects was more glycosylated with sialic acid than the recurrent stone

formers. The terminal sialic acid is essential for the inhibitor function of THP in healthy subjects (Kumar S*et al*., 1990). Therefore, carbohydrate structure of THP is the major feature that may regulate kidney stone formation. A loss of several S–S bridges by oxidation could cause alterations in the secondary structure of the protein.

Scanning electron microscopy analysis

Fig. 7 and 8 represents the SEM analysis of normal and diseased samples. More aggregation was observed in THP of diseased sample compared to that of normal samples which is correlated with the fact that that at low pH, high calcium, high IS, THP self-aggregates (Hess B*et al*., 1993) which lowers its inhibitory activity.

Polyclonal antibody production

Ouchterlony double diffusion method was performed to confirm the production of polyclonal antibody production. Polyclonal antibodies were loaded into wells 2, 3, 4, 5 and 6 with first

well as control. Purified THP antigen produced was loaded in well 7 and line of precipitation was observed around the well. Hence production of polyclonal antibody against THP was confirmed.

CONCLUSION

In this study, out of the four methods used for isolating THP protein, ammonium sulphate and sodium chloride methods gave prominent bands. The protein to Creatinine ratio of normal and diseased samples was calculated in order to determine abnormality in diseased patients. UV Spectrometry analysis was done for both normal and diseased samples to determine concentration of protein present. SEM images of normal and diseased revealed that aggregation of THP was more in diseased samples. The purified THP was injected into Balb/C mice and production of polyclonal antibody was confirmed by ODD method. In future, polyclonal antibodies produced from THP will be tagged with HRP conjugate and may be used as an earlier marker and hence a good diagnostic tool for the renal diseases to be analyzed.

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