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ISOLATION OF NORMAL BUFFALO URINARY PROTEINS

M. RAGA SUDHA*, PARVATHALA KALYANI AND VASILI ASHOK

Dept. of veterinary biochemistry, college of veterinary science, sri venkateswara veterinary university, rajendranagar, hyderabad, andhra pradesh, india.

ABSTRACT

Uromodulin (85-90 kDa) and uroplakins (15-47 kDa) are normal urinary proteins produced by thick ascending limb of Henle's loop of kidney tissue and urinary bladder respectively. Tissue specificity of these proteins facilitates the idea of transforming urinary system into a bioreactor. Identification of their promoter sequences helps in the construction of a transgene to express foreign proteins in the urine. High buffalo population and large volumes of urine voided by them, make them a better choice as model animals for transgenic research. The normal urinary proteins were precipitated by three precipitating agents viz., acetone, ammonium sulphate and sodium chloride. Analysis of protein precipitates by SDS PAGE revealed a prominent high molecular weight band at the level of 85 kDa in Coomassie stained gels. It is selectively precipitated by sodium chloride and it could be uromodulin. The inconsistent 50kDa band identified in acetone and ammonium sulphate precipitated samples in Coomassie stained gel and low molecular weight protein bands (27 kDa & 15 kDa) identified in silver nitrate stained gel could be uroplakins. The normal urinary protein precipitate concentrations with respect to the total urinary proteins were identified to be 1.1%, 0.156% and 0.2% for acetone, ammonium sulphate and sodium chloride precipitated samples respectively.

KEY WORDS : Bioreactor, Promoter, transgenic research, Uromodulin, Uroplakins.

M. RAGA SUDHA

Dept. of veterinary biochemistry, college of veterinary science, sri venkateswara veterinary university, rajendranagar, hyderabad, andhra pradesh, india.

INTRODUCTION

Advances in genetic engineering and transgenic animal technology enhanced the scope to produce large quantities of economically valuable proteins[1,2]. In recent years the idea of transforming urinary system into a bioreactor and use of urine as an expression system is gaining importance owing to the presence of minute quantities of certain proteins like uromodulin and uroplakins in physiological urine. When compared to mammary gland based bioreactor, it has advantages like ease of protein purification, animals of both sexes can be used and animal age starting from day old can be used[3, 4, 5].

Uromodulin is the most abundant protein (85 kDa) in the normal human urine[6]. It is a glycosylphosphatidylinositol (GPI) anchor linked membrane protein exposed luminally in the cells of thick ascending limb of henle's loop and distal convoluted tubule. It is excreted into the urine due to cleavage by the enzymes like proteases and GPI-specific phospholipase[7].

Uroplakins are the integral membrane proteins that transform the apical surface of urothelial cells into a specialized asymmetric unit membrane (AUM) with urothelial plaques. These plaques are formed by four uroplakins namely uroplakin Ia, Ib, II and III[8]. They are excreted into the urine due to the continuous membrane regeneration of urinary bladder during its adaptation to changes in the urine volume[9].

Isolation of these proteins and identification of their promoter sequences enables the construction of a transgene using these promoters so that a foreign protein can be expressed in normal urine.

Buffaloes are a better choice for transgenic research because, India is a country with world's largest buffalo population and also because research in buffaloes is still in its infancy. They void large volumes of urine viz., 20 L/day.

So in the present study, buffaloes are chosen as model animals for the isolation of normal urinary proteins. It is a preliminary study with the long term goal of producing a transgenic buffalo which can express foreign proteins in the urine.

MATERIALS AND METHODS

Urine samples (500 ml) were collected from ten apparently healthy, lactating Murrah buffaloes and stored at 4^0C. Individual urine samples were used for quantification of proteins and pooled urine samples were used for gel analysis to avoid variations between different animals.

PRECIPITATION AND CENTRIFUGATION

The urine samples were precipitated with three different protein precipitating agents viz., acetone, ammonium sulphate and sodium chloride of analytical grade obtained from Qualigens.

PRECIPITATION WITH ACETONE

Equal quantities (7 ml each) of urine and cold acetone (-20^0C) were taken in centrifuge tubes and mixed thoroughly[10]. These samples were centrifuged at 2500g for 30 minutes at 4^0C in an Eppendorf 5810R refrigerated

centrifuge. Acetone was decanted and the sediments were air dried. The sediments were carefully dissolved in 200 ml of phosphate buffer saline (PBS: p^H 7.4, 27.6 gm/L of NaH_2PO_4, 28.4 gm/L of Na_2HPO_4, 0.5 mg/ml of EDTA, 17 µg/ml of PMSF, 0.05% v/v of Tween 20, 0.02% of Sodium azide) and preserved at -20^0C for gel analysis.

PRECIPITATION WITH AMMONIUM SULPHATE

160 gms of ammonium sulphate was added to 250 ml of urine to obtain 90% saturation at which urinary proteins precipitate[11]. The addition of ammonium

sulphate was performed on a magnetic stirrer at 4^0C and the samples were preserved at 4^0C for 24 hrs with sodium azide (0.02%) as preservative. Then they were centrifuged at 2500g for 30 minutes at 4^0C. The sediments were dissolved in 100ml of phosphate buffer saline and preserved for dialysis.

PRECIPITATION WITH SODIUM CHLORIDE

Urine was precipitated with sodium chloride at a final concentration of 0.29mol/L[12, 13]. Then the contents were stirred and preserved at 4^0C for 48hrs with sodium azide (0.02%) as preservative. Then they were centrifuged at 2500g for 30 minutes at 4^0C. The sediments from 15 ml solutions were dissolved in 100 ml of phosphate buffer saline and preserved for dialysis.

DIALYSIS

Ammonium sulphate and sodium chloride precipitated samples were dialyzed against dialysis buffer (PBS) and preserved at -20^0C for freeze drying.

LYOPHILIZATION

Frozen samples were lyophilized at 100 millitorr pressure using Virtis benchtop lyophilizer. These lyophilized samples were dissolved in required quantities of PBS and used for gel analysis.

SDS-PAGE

The individual protein precipitates of 10 animals were pooled for each of the three precipitation methods and were analyzed by SDS PAGE with 5% stacking gel and 12% resolving gel using Banglore genei apparatus according to the method described by Bollag et al (1996)[10]. The gel was stained by Coomassie brilliant blue method[10] and silver nitrate method[14].

PROTEIN ESTIMATION

Quantification of protein in fresh urine and precipitated urine samples of ten healthy buffaloes was done using Lowry assay with bovine serum albumin as a standard[15].

RESULTS AND DISCUSSION

Analysis of Coomassie brilliant blue stained SDS PAGE gel revealed a consistent high molecular weight 85kDa protein band in acetone, ammonium sulphate and sodium chloride precipitated urine samples (Fig. 1). Sodium chloride precipitated sample did not reveal any other protein bands except the 85kDa protein band which is very prominent, while acetone and ammonium sulphate precipitated samples revealed other faint bands of 66kDa and 50 kDa which were inconsistent. Ammonium sulphate precipitated samples revealed some more inconsistent protein bands in the medium molecular weight range. The banding pattern in acetone and sodium chloride precipitated samples with or without β - mercaptoethanol was similar (Fig. 1).

Tamm & Horsfall (1952) isolated Human Tamm Horsfall protein (THP) / uromodulin using sodium chloride at a concentration of 0.58 mol/lit[16]. Roger (1987) & Kobayashi (2001) isolated an 85-90kDa protein from human urine selectively by sodium chloride precipitation method and identified it as uromodulin[12, 13]. Fontan *et al.* (1994)[17] isolated a 92kDa protein band from human urine sample by sodium chloride precipitation, ultracentrifugation and DEAE affigel blue chromatography and identified it as uromodulin. Serafini-Cessi *et al.* (1989) isolated 85kDa protein by an alternative method using diatomaceous earth filter which retained most of the protein in human urine and identified it as uromodulin[18]. In the present study, the 85kDa protein is very prominent and is the only protein band identified in sodium chloride precipitated samples although it is also found in ammonium sulphate and acetone

precipitated samples. So it could be uromodulin. It is consistently seen in all the three precipitation methods which indicate that it is a normal urinary protein. The protein uromodulin was found to be evolutionarily conserved in vertebrates[19].

We got similar banding pattern with and without β- mercaptoethanol (Fig. 1) in acetone and sodium chloride precipitated samples. But Daniella *et al.* (2001) reported that under reducing conditions, the uromodulin (THP) was identified as high molecular weight band compared to non reducing conditions[7]. This phenomenon was explained as that when such a large number of S-S bridges (predicted 48) are reduced, the THP molecule acquires a more linear configuration, with a consequent increment in the SDS binding and a decrease in the SDS-PAGE motility.

Analysis of silver nitrate stained gel (Fig. 2) revealed some medium and low molecular weight protein bands in acetone and ammonium sulphate precipitated samples and very faint medium and low molecular weight protein bands in sodium chloride precipitated samples which are inconsistent. Among them, two prominent low molecular weight protein bands of 27kDa and 15kDa are identified in all the three protein precipitates of urine (Fig 2).

The medium molecular weight protein band of 50kDa and low molecular weight protein bands of 27 & 15kDa could be uroplakins[8, 9, 20]. Uroplakin III (47kDa) was detected in the human urine samples by immunoblot analysis by Born *et al* (2003)[9]. Uroplakins I, II & III with molecular weights of 27-28kDa, 15kDa and 47kDa respectively were isolated by SDS PAGE from purified asymmetric unit membrane of bovine urothelium by Wu *et al.* (1990)[20]. Precipitation of uroplakins from the urine using protein precipitating agents is not performed till now.

The other medium and low molecular weight protein bands identified in acetone and ammonium sulphate precipitated samples could be due to proteins entering the urine from serum, renal tubular cells, epithelial cells of the urinogenital tract and glandular secretions of urinogenital tract[21]. They could be transporters, adhesion molecules, complement, chaperones, receptors, enzymes, serpins, cell signaling proteins and matrix proteins visvualized by 2D-PAGE as reported by Visith Thongboonkerd *et al.* (2002)[22]. Acetone precipitated more acidic and hydrophilic proteins[22].

Medium molecular weight protein bands in acetone precipitated samples (66, 50 kDa), ammonium sulphate precipitated samples (66, 50, 38 kDa) and a number of low molecular weight faint bands (not observed in this gel picture) were inconsistent due to dilution effect of liquid intake by the animal, diet, temperature, climate and other physiological factors which are yet to be ruled out.

From the present study, it can be concluded that sodium chloride is the best precipitating agent as it selectively precipitates uromodulin (85kDa). Ammonium sulphate is required in large quantities for precipitation when compared to that of sodium chloride. Acetone precipitation should be done carefully otherwise it causes protein denaturation[23].

QUANTIFICATION

Quantification of total protein content in fresh urine and precipitated samples (acetone, ammonium sulphate and sodium chloride) of urine was done to find out a rough estimate of the amount of foreign protein that can be expressed in the bovine urine.

The total protein concentration in bovine urine was found to be 956.7±10.6 µg/mL. The total protein concentration in cattle urine estimated by Lowry method was reported to be 626 ± 48.7 µg/mL by Weeth *et al.* [24] & 172 ± 26 µg/mL by Kerr *et al.* [5]. The higher value in bovine urine could be due to species variation or dilution effect of the urine.

Acetone yielded a significantly high amount of protein precipitate (10.60±0.39 µg/mL) when compared to ammonium sulphate (1.50±0.04 µg/mL) and sodium chloride (1.91±0.13 µg/mL). But the banding pattern in electrophoresis is not commensurate with the protein concentration values in acetone precipitated sample. This could be due to the precipitation of some salts and other metabolic waste components along with the protein by acetone, which might have positively influenced Lowry assay[25]. Ammonium sulphate and sodium chloride precipitated almost equal amounts of protein. The recovery yield of the three precipitation methods viz., acetone, ammonium sulphate and sodium chloride in isolating buffalo urinary proteins was found to be 1.1%, 0.156% and 0.2% of the total protein in fresh urine.

Achiraman & Archunan (2002)[26] reported the urinary protein concentration in ammonium sulphate precipitated samples at 60% saturation to be 1.64 and 1.37mg/ml in bull and cow respectively. These values are very high when compared to 1.50±0.04 µg/mL of protein estimated in ammonium sulphate precipitated sample at 90% saturation in the present study. The higher protein values obtained in bulls and cows as compared to buffaloes could be due to species variation and difference in the ammonium sulphate saturation at which precipitation was carried out.

Visith Thongboonkerd *et al*. reported that the recovery yield of human urinary proteins by ammonium sulphate precipitation at 90% saturation was 11% and by acetone precipitation at 50% saturation was 25%[27]. But in the present study, the recovery yield of buffalo urinary proteins by ammonium sulphate precipitation at 90% saturation was found to be 0.156% and by acetone precipitation at 50% saturation was found to be 1.1%. Hence, it can be concluded that recovery yield of buffalo urinary proteins by ammonium sulphate and acetone precipitation methods is very less when compared to that of human urinary proteins. There is no single protocol to precipitate all the proteins present in the urine sample[27]. Only a fraction of total urinary proteins can be precipitated by a given method. Among the three methods used in the present study, sodium chloride precipitation is found to precipitate uromodulins selectively.

Uromodulin levels in humans were reported to be in the range of 22-140 mg/24hours[28, 29]. The estimated uromodulin values in buffaloes in the present study is 38.2 ± 2.6 mg/24hrs (sodium chloride precipitated urine sample), which was far less when compared to humans. This could be due to alkaline pH of bovines which might require less uromodulin as protective protein.

Kerr *et al.* (1998) reported that the transgenic protein content in urine of farm animals could represent 0.1 to 1.0% of the total urinary protein[5]. It correlates with uromodulin concentration in sodium chloride precipitated sample in buffalo urine in the present study.

A number of methods for quantification of THP in urine other than ammonium sulphate precipitation and Lowry assay were performed using radial electroimmunodiffusion assay[30], radioimmunoassay[31] and enzyme linked immunoassay[32], where major obstacle is gel forming ability of THP[12].

CONCLUSION

Our research work reveals that uromodulin (85kDa) is the prominent normal protein produced by buffaloes as found in Coomassie blue staining by all the three precipitation methods. Uroplakins (50, 27 & 15kDa) were found as faint and inconsistent bands in Coomassie and Silver nitrate staining methods.

Their identity can be confirmed by immunohistochemistry and western blotting. Further the present work paves way for long

term goal of enabling buffaloes as bioreactors for the expression of foreign proteins in urine using uromodulin (85kDa) promoter as uromodulin was identified as prominent normal urinary protein.

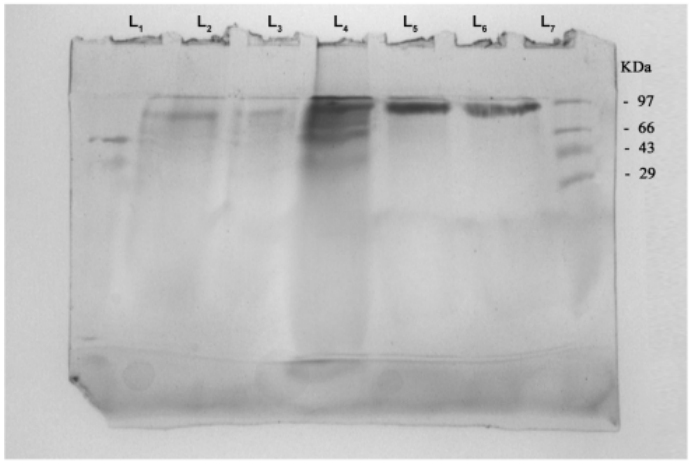

FIG 1

SDS PAGE of protein precipitates of urine samples (Coomassie brilliant blue stained gel)

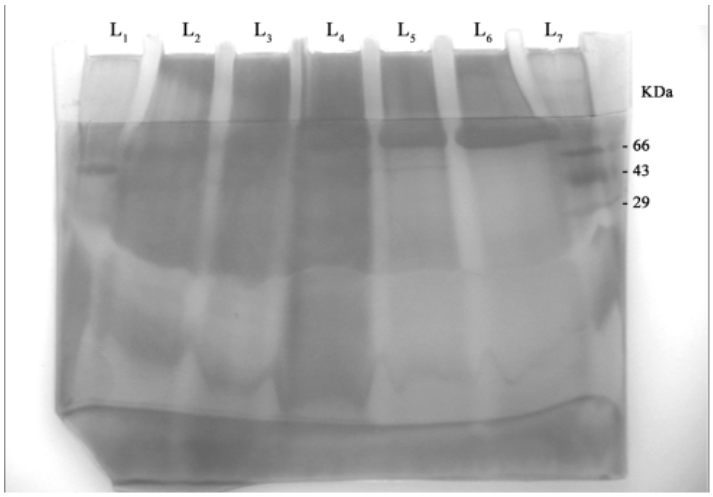

FIG 2

SDS PAGE of protein precipitates of urine samples (Silver nitrate stained gel)

FIGURE LEGEND

FIG 1: SDS-PAGE of protein precipitates of urine samples (Coomassie brilliant blue stained gel) (L1: Bovine serum albumin, L2: Acetone precipitated sample without β mercaptoethanol, L3: Acetone precipitated sample with β mercaptoethanol, L4: Ammonium sulphate precipitated samples, L5: Sodium chloride precipitated sample without β mercaptoethanol, L6: Sodium chloride precipitated sample with β mercaptoethanol, L7: Molecular weight markers)

FIG 2: SDS-PAGE of protein precipitates of urine samples (Silver nitrate stained gel) (L1: Bovine serum albumin, L2: Acetone precipitated sample without β mercaptoethanol, L3: Acetone precipitated sample with β mercaptoethanol, L4: Ammonium sulphate precipitated samples, L5: Sodium chloride precipitated sample

without β mercaptoethanol, L6: Sodium chloride precipitated sample with β mercaptoethanol, L7: Molecular weight markers)

TABLE 1
COMPARISON OF PROTEIN CONCENTRATION IN PRECIPITATED URINE SAMPLES.

Precipitating method	Protein (PC) (g/ml)	Creatinine (CC) (mg/ml)	PC/CC (g/mg)	Protein (mg/20liters)	%/total Urinary protein
Acetone	10.600.39	0.860.09	13.391.14	212.127.63	1.10
Ammonium Sulphate	1.500.04	0.860.09	1.920.20	29.910.79	0.156
Sodium Chloride	1.910.13	0.860.09	2.360.18	38.292.63	0.2
Urine	956.7±10.6	0.860.09	1227.7±121	19000.13±0.2	

Values are mean ± SE of 10 observations.

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