

α_2 - μ -Globulin Fragment (A2-f) from Kidneys of Male Rats forms Multimers *in Vitro*

Abdul Hai, Nadeem A. Kizilbash, and Jamal Alruwaili

Abstract— α_2 - μ -Globulin fragment (A2-f) functions as a fatty acid binding protein in kidneys of male rats. It has 100% sequence homology with amino acids 10-160 of α_2 - μ -Globulin (A2), an 18.6-kDa protein that is synthesized in male rat liver and is present in urine. A2-f is produced from A2 by the removal of 3 residues from the N-terminus and 9 amino acids residues from the C-terminus. This project aimed to isolate and purify A2-f (to >90% purity) for NMR analysis since the solution state structure of A2-f is still unknown. The purification failed due to formation of multimers *in vitro* but it did reveal heterogeneity of this protein as isolated from tissue.

Index Terms—2- μ -Globulin fragment (A2-f), 2- μ -Globulin (A2), kidney fatty acid binding protein, Lipocalins, protein aggregation, male rat urinary proteins.

I. INTRODUCTION

α_2 - μ -Globulin (A2) is synthesized in the liver, secreted in blood and excreted in the urine of male rats. The protein has been reported to be absent in plasma and urine of female rats [1]. α_2 - μ -Globulin fragment (A2-f) is a proteolyzed fragment of A2 (Fig. 1) [2]. It has a molecular weight of 15.5 kDa and it binds long chain fatty acids *in vitro* with a 1:1 stoichiometry and a very high affinity (K_d ranges from 0.1-2 μ M) [3]. Metabolic data, obtained from suspensions of fresh proximal tubule segments, has shown that A2-f facilitates fatty acid oxidation and promotes ATP production in the kidneys [4]-[7]. It is believed that unusual structural features permit A2-f to be targeted to the proximal tubule cell, to escape lysosomal degradation and to enter the cytosol. A2-f was initially thought to be a unique kidney fatty acid binding protein for several reasons: 1) it has a molecular weight similar to all known FABPs; 2) it is found in the soluble protein fraction; 3) it is abundant in cells with high fatty acid flux; and 4) it binds long chain fatty acids *in vitro* [3]. Localization of A2-f to the proximal tubule, the site with the highest fatty acid flux in the kidney, further supported the idea that the 15.5 kDa protein is a unique kidney fatty acid binding protein [3].

A2-f is known to be a member of the Lipocalin family of proteins [8]. The precursor protein, A2, is also a member of this family. The amino acid sequence of A2 was earlier

determined using a combination of nucleic acid and protein sequencing techniques [9]. The Lipocalin family of proteins is very diverse at the sequence level yet displays highly conserved structures. Twelve structures of distinct members of this protein family have been solved experimentally [10]. These structures show that most Lipocalins share three characteristic conserved sequence motifs (Kernel Lipocalins) while others are more divergent (Outlier Lipocalins). The Lipocalins are also part of a larger protein superfamily, the Calycins (which includes the fatty acid binding proteins), Avidins (a group of metalloproteinase inhibitors) and Triabin. The Lipocalin superfamily is characterized by structural homology (repeated +1 topology β -barrel) and by conservation of a structural signature [10]. The binding property of this family of proteins for small hydrophobic molecules, and to a lesser extent, to cell surface receptors, is well known, it is also known that formation of macromolecular complexes is also a common feature of this family [10]. This project employed standard techniques of protein purification to isolate and purify A2-f (a member of Lipocalin superfamily) for NMR analysis since the solution state structure of A2-f is still unknown.

II. MATERIALS AND METHODS

Sephadex G-75 packing material was purchased from Pharmacia LKB Biotechnology Inc. Both DE52 and CM52 resins were purchased from Whatman, Inc. Molecular weight standards for SDS Page gels were purchased from Biorad. Male Sprague-Dawley rat kidneys obtained were from Taconic Farms. The kidney tissue was minced and homogenized in 100 mM phosphate buffer at pH 7.2, containing 1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF), a protease inhibitor and 0.02% NaN_3 . The homogenate was centrifuged at 5000 \times g (SS-34 rotor at 3000 rpm) for twenty minutes at 4 $^\circ\text{C}$. The supernatant was then re-centrifuged at 100,000 \times g for ninety minutes (26,500 rpm). The layer of fat on the top of each centrifugation tube was removed by suction after each centrifugation step. The supernatant was then applied to a Sephadex G-75 column (2.5 \times 90 cm) with a bed volume of 1.5 liters and eluted with 0.03 M Sodium Borate buffer at pH 8.5. Fractions containing the protein of interest (identified by Western blots using rabbit polyclonal anti-bodies) which eluted between an elution volume of 1.162-1.242 L (K_{av} = 0.48) were collected and pooled together and adjusted to 0.03 M Sodium Borate, 5mM Sodium Chloride at pH 8.5. This fraction was applied to a DE-52 column (1.5 \times 20 cm) equilibrated with 0.03 M Sodium borate, 5mM Sodium Chloride at pH 8.5. Adsorbed protein was eluted with 150 mL of a linear salt gradient of 5-300 mM

Manuscript received August 29, 2013; revised December 5, 2013.

Abdul Hai and Nadeem A. Kizilbash are with the Department of Biochemistry, Faculty of Medicine & Applied Medical Sciences, Northern Border University, Arar-91431, Saudi Arabia (e-mail: synavia@gmail.com, fsd707@gmail.com).

Jamal Alruwaili is with Faculty of Medicine & Applied Medical Sciences, Northern Border University, Arar-91431, Saudi Arabia (e-mail: malkh1195@hotmail.com).

NaCl, 0.03 M Sodium Borate, pH 8.5. Fractions identified as containing the protein of interest (identified by Western blots) were pooled together and dialyzed overnight against de-ionized water at 4°C. The pH of dialysate was adjusted by 0.01 M Sodium Acetate at pH 5.0, before it was applied to a CM-52 column (0.9×4 cm) equilibrated with 0.01 M Sodium Acetate at pH 5.0. The column was washed with 10 mL of this buffer and then the protein was eluted with 50 mL gradient of 0.01-0.5 M Sodium Acetate at pH 5.

EEASSTRGNLDVAKLNGDWFSIVVASNKREKREENGSMRVFMQ

HIDVLENSLGFKFRIKENGECRELYLVAYKTPEDGEYFVEYDGG

NTFTILKTDYDRYVMFHILNFKNGETFQLMVLYGRTKDLSSDIK

EKFAKLCEAHGITRDNIDLTIKTRCLQARG

Fig. 1. Amino Acid sequence of A2 (residues shown underlined are cleaved to form A2-f) [3].

III. RESULTS

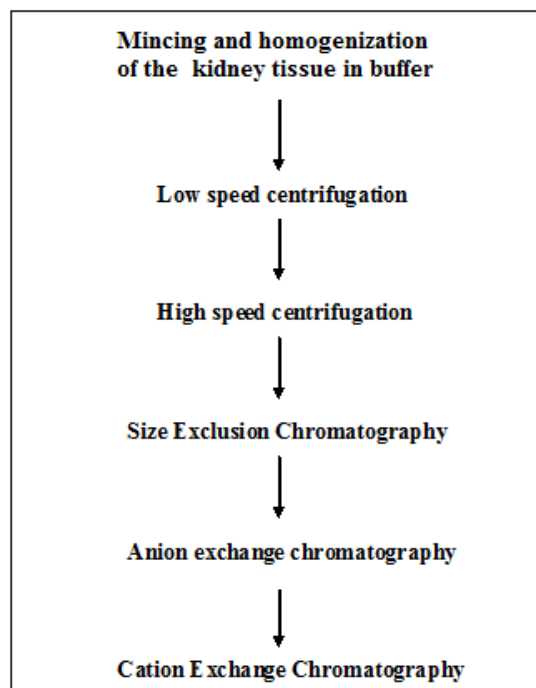
A2-f was isolated and purified from 100 male rat kidneys using established biochemical techniques [3]. The methodology used is summarized in Schematic 1. Frozen male rat kidneys were purchased from Taconic Farms using male Sprague-Dawley rats weighing 150-250 g. The kidneys were placed in ice cold 100 mM phosphate buffer at pH 7.2, containing 1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF), a protease inhibitor and 0.02% Sodium Azide. The kidneys were cleaned by removal of adhering fat and renal capsules. All subsequent steps were performed at 4 °C and all buffer pH values for solutions were measured at 4 °C. The tissues were minced with a single-edge razor blade and diluted to twice the volume by 0.1 M phosphate buffer. The solution was then homogenized with a polytron homogenizer for thirty seconds at 50% power. The homogenate was centrifuged at 5000×g (SS-34 rotor at 3000 rpm) for twenty minutes at 4 °C. The supernatant was then re-centrifuged at 100,000×g (26,500 rpm) for ninety minutes. The layer of fat on the top of each centrifugation tube was removed by suction after each centrifugation step. The total protein concentration in the 180 mL supernatant was estimated to be 7.1 g by use of a Biorad assay. A calibration curve was constructed by measuring the absorbance of BSA solutions of known concentrations at 595 nm by a UV-Vis spectrophotometer. Five standard solutions were used with concentrations that ranged from 0.0625 –1.0 mg/mL of Bovine Serum Albumin (BSA). The concentration of the sample was then estimated from this curve.

Twenty five mL of the supernatant was then applied to a Sephadex G-75 Size Exclusion column (2.5×90 cm) with a bed volume of 1.5 liters and eluted with 0.03 M sodium borate, pH 8.5 at a flow rate of 1.3 mL/minute. 22.5 mL fractions were collected in 25 mL test tubes, which were stored at 4 °C (Fig. 2).

Fractions 62-97, identified by Western blots as containing the protein of interest, were pooled together. The buffer concentration was adjusted to 0.03 M Sodium Borate, 5mM Sodium Chloride. These pooled fractions were applied to a DE-52 column (1.5 × 20 cm) equilibrated with 0.03 M

Sodium Borate, 5 mM NaCl at pH = 8.5. The protein was eluted by 150 mL of a linear salt gradient of 5-300 mM Sodium Chloride, 0.03 M Sodium Borate at pH 8.5 (Fig. 3).

Fractions 18-30 identified as containing the protein of interest by Western gels were pooled together and dialyzed overnight against de-ionized water at 4°C. The dialysate was adjusted to pH 5.0 by 0.01 M Sodium Acetate, before it was applied to a CM-52 column (0.9×4 cm) pre-equilibrated with 0.01 M Sodium Acetate at pH 5.0. The column was washed with 10 mL of 0.01 M Sodium Acetate at pH 5.0 and then the protein was eluted by a 50 mL gradient of 0.01-0.5 M Sodium Acetate at pH 5.0 (Fig. 4). An analysis of the fractions isolated from Size Exclusion chromatography by 12% SDS PAGE gel is shown Fig. 5. It revealed more than thirteen bands for the purified fractions. Apparently, the protein had aggregated after Size Exclusion Chromatography.



Schematic 1. Protocol used for purification of A2-f from kidneys [3].

IV. DISCUSSION

The purification was unsuccessful for a number of reasons. These reasons are:

- 1) A typical purification scheme for a non-enzymatic protein isolated from a tissue is shown in Schematic 2. The purification scheme used by Lam *et al.* [3] actually used the reverse order of chromatographic purification. Size exclusion chromatography was performed before Ion-exchange chromatography.
- 2) Rat kidney is marked by the presence of numerous proteins. Hemoglobin is the most abundant protein present in the kidney. It has a molecular weight of about 14 kDa which is comparable with the molecular weight of A2-f (15.5 kDa). The kidney tissue used for purification needs to be perfused so as to remove the hemoglobin present in the kidney tissue.
- 3) The paper used for this purification [3] (Lam *et al.*, 1988) omitted to mention that A2-f possesses a Cys-129 residue which has a free sulfhydryl group (Fig. 6) [11].

Cys-129 can cross-react with similar Cysteines with free Sulfhydryl groups to form disulfide bonds. One way to avoid aggregation is to add 1mM dithiothreitol (DTT) to the buffers used for centrifugation and size exclusion chromatography [12].

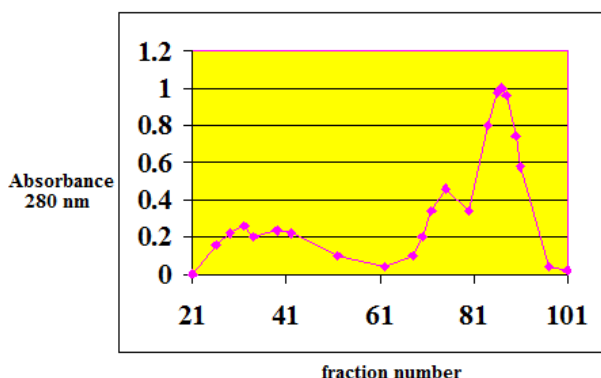


Fig. 2. Size exclusion chromatography of homogenized kidney tissue using a 0.03 M Sodium Borate buffer for elution at pH 8.5 and a sephadex G-75 column (2.5×90 cm).

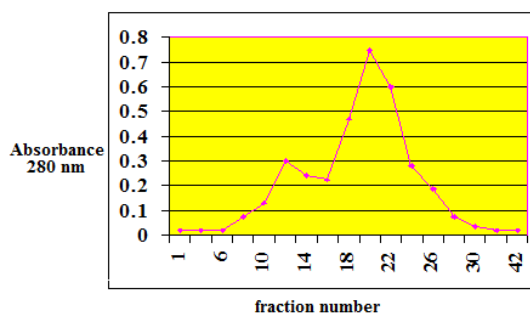


Fig. 3. Anion exchange chromatography using linear gradient of 0.03 M Sodium Borate, 5 mM NaCl to 0.03 M Sodium Borate, 300 mM NaCl at pH = 8.5 and a DE 52 column (1.5×20.0 cm).

A Japanese group has reported a successful purification of this protein [12]. In their protocol, 1 mM Dithiothreitol (DTT) was present in all the preps. They have reported that the protein eluted and was purified 9.3-fold by sequential gel filtration and anion-exchange chromatography. Homogeneity was shown by a single band on polyacrylamide gel with a molecular weight of about 15.5 kDa. The binding of Palmitic Acid to A2-f was found to be saturable. Examination of fatty acid binding revealed the presence of a single class of fatty acid-binding sites. The apparent K_d was 1.0 μ M and the maximal binding capacity was 48 nmol of fatty acid/mg of protein. The protein showed similar binding characteristics and a single band was observed by Western gel analysis.

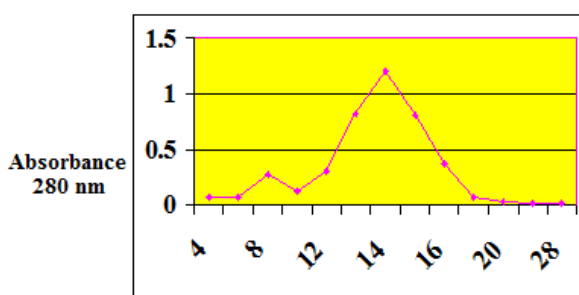


Fig. 4. Cation exchange chromatography using a 50 mL linear gradient of 0.01-0.05 M Sodium Acetate at pH = 5.0 and a CM 52 column (0.9×4.0 cm).

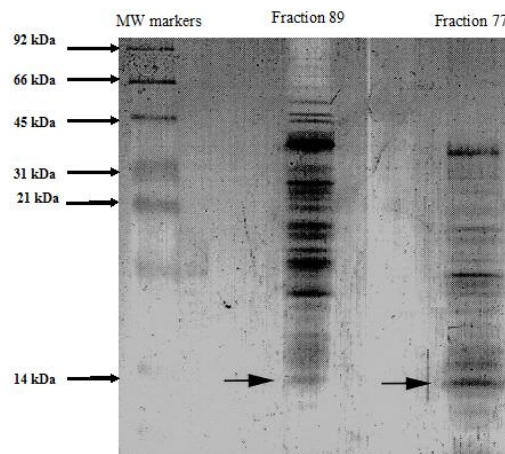


Fig. 5. 12% SDS PAGE gel of fractions 77 and 89 from G-75 size exclusion chromatography. The bands at 42 kDa and 28 kDa show the aggregated protein due to imperfect purification.

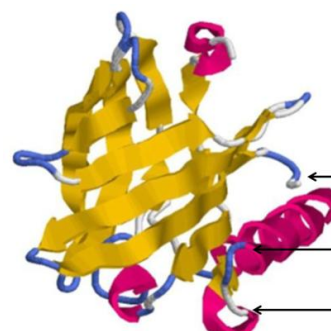


Fig. 6. Tertiary structure of A2-f showing the position of N-terminus, Cys-129 and C-terminus respectively (shown by arrows). Cys-129 is at the end of α -helix IV [11].

V. CONCLUSION

The purification conducted for A2-f, failed to provide >1 mg amount of purified protein but it did help shed some light on the experimental protocols discussed in literature. For instance, this project showed that the paper published by Lam *et al.* [3] suffers from some serious shortcomings. The paper never mentioned that the primary source for the experimental protocols used was an already published paper by Fujii *et al.* [12]. Even when it did cite the latter-mentioned paper, it cited it as the source for physiological conclusions rather than experimental protocols.

REFERENCES

- [1] G. Vandoren, B. Mertens, W. Heyns *et al.*, "Different Forms of $\alpha_{2\mu}$ -Globulin in Male and Female Rat Urine," *Eur J Biochem.*, vol. 134, no. 1, pp. 175-181.
- [2] H. Kimura, S. Odani, J. Suzuki, M. Arakawa, and T. Ono, "Kidney fatty acid-binding protein: Identification as $\alpha_{2\mu}$ -globulin," *FEBS Letters*, vol. 246, pp. 101-104, 1989.
- [3] K. T. Lam, S. Borkan, K. P. Claffey, J. H. Schwartz, A. V. Chobanian, and P. Brecher, "Properties and differential regulation of two fatty acid binding proteins in the rat kidney," *J Biol Chem.*, vol. 263, pp. 15762-15768, 1988.
- [4] S. C. Borkan, Y. H. Wang, K. T. Lam, P. Brecher, and J. H. Schwartz, "Hepatic alpha 2 mu-globulin: a potential metabolic role in the rat proximal tubule," *Am J Physiol.*, vol. 271, no. 3, pp. F527- F538, 1996.
- [5] Y. H. Wang, and S. C. Borkan, "Prior heat stress enhances survival of renal epithelial cells after ATP depletion," *Am J Physiol.*, vol. 270, pp. F1057- F1065, 1996.
- [6] S. C. Borkan, Y. H. Wang, W. Lieberthal, P. R. Burke, and J. H. Schwartz, "Heat stress ameliorates ATP depletion-induced sublethal injury in mouse proximal tubule cells," *Am J Physiol.*, vol. 272 no. 3, pp. F347- F355, 1997.

- [7] Y. Wang, M. A. Shia, T. G. Christensen, and S. C. Borkan, "Hepatic alpha 2 microglobulin localizes to the cytosol of rat proximal tubule cells," *Kidney Int.*, vol. 57, no. 3, pp. 1015-1026, 2000.
- [8] D. R. Flower, "The lipocalin protein family: a role in cell regulation," *FEBS Letters*, vol. 354, pp. 7-11, 1994.
- [9] R. D. Unterman, K. R. Lynch, H. L. Nakhasi, K. P. Dolan, J. W. Hamilton, D. V. Cohn, and P. Feigelson, "Cloning and sequence of several $\alpha_2\mu$ globulin cDNAs," *Proc. Natl. Acad. Sci.*, USA, vol. 78, PP. 3478-3482, 1981.
- [10] D. R. Flower, A. C. North, and C. E. Sansom, "The lipocalin protein family: structural and sequence overview," *Biochim Biophys Acta*, vol. 1482, no. 1-2, pp. 9-24, 2000.
- [11] A. Hai, and N. Kizilbash, " $\alpha_2\mu$ -Globulin fragment (A2-f) from Kidneys of Male Rats," *Bioinformation*, vol. 9, no. 3, pp. 145-149, 2013.
- [12] S. Fujii, H. Kawaguchi, and H. Yasuda, "Purification and characterization of fatty acid-binding protein from rat kidney," *Archives of Biochemistry & Biophysics*, vol. 254, no. 2, pp. 552-558, 1987.



Abdul Hai was born in Allahabad, India. He received his master's degree from University of Allahabad, India. His doctoral degree was awarded in Biochemistry in 2002 from IIT-Kanpur in India. He is presently working as an assistant professor of Biochemistry at Northern Border University in Arar, Saudi Arabia. He has previously worked as an Assistant Professor at Garyounis University in Libya. His work experience also includes postdoctoral fellowship at Max Planck (Germany) and University of North Carolina (Raleigh, U.S.A.). He has published eight papers in international journals on topics as diverse as Proteomics, Protein Structure, Drug Delivery and Molecular Docking.



Nadeem Kizilbash was born in Karachi, Pakistan. He received his bachelor's degree in chemistry in 1989 from Longwood College in Farmville, U.S.A. His master's degree was also in Chemistry in 1994 from Washington University in St. Louis, U.S.A. His doctoral degree was awarded in Biophysics in 2003 from Boston University in U.S.A. He is presently working as an assistant professor of Biochemistry at Northern Border University in Arar, Saudi Arabia. He has previously worked as an Assistant Professor at Quaid-i-Azam University in Pakistan. His work experience also includes service as a Senior Scientist at National Institute for Biotechnology and Genetic Engineering in Pakistan. He has published fifteen papers in various journals on topics as diverse as Proteomics, Protein Structure, Drug Delivery and Gene Polymorphism. Dr. Kizilbash is currently a member of editorial board of three international research journals.



Jamal Alruwaili was born in Arar, Saudi Arabia. He received his doctoral degree in Biochemistry in 2011 from the University of Portsmouth in U.K. The topic of his dissertation was: "Serum Proteomic Analysis of Prostate Cancer Progression". He is presently working as the Vice Dean of the Faculty of Applied Medical Sciences at Northern Border University in Arar, Saudi Arabia. He has previously worked as a scientist at King Faisal Specialist Hospital Hospital in Riyadh, Saudi Arabia. He has published papers in international journals on topics as diverse as Proteomics, Protein Structure, Molecular Docking and Drug Delivery.