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Extraction, purification and characterization of crystallin protein of cataractus eye lens nucleus

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Abstract

The purpose of this study is to separate and identify the crystallin protein present in nucleus of human cataractous eye lens. Cataractous lenses were collected from different eye hospital for the patients of different etiologies having ages between 40 to 80 years. Lens nucleus proteins were extracted into four fractions on the basis of their solubility in different media by applying a reported method. These fractions were buffer soluble proteins (PS), urea soluble proteins (PU), yellow fraction proteins (PY) and insoluble proteins (PI). All three soluble fractions were subjected to HPLC and GPC analysis. Both HPLC and GPC analysis shows that each fractioncontains α, β and γ-crystallins, a major class of protein presents in the lens of vertebrates. Various chromatographic parameters including precision, accuracy and linearity have been evaluated. Studies of water-insoluble crystallins using sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) have demonstrated extreme homogeneity with evidence of major components with molecular masses of 18-70 KDa, similar to the crystallin of water-soluble portion.The method was found to be suitable for the analysis of various isomers of crystallin protein present in human cataractous eye lens nucleus. The detailed results of GPC are discussed. This study provided first time the HPLC and GPC analysis of human cataractous eye lens nucleus.

***Keywords*:** Cataractous lenses, HPLC analysis, GPC analysis, Gel electrophoresis, Pathological analysis

INTRODUCTION

Cataract is the loss of lens transparency which usually affects more than 20 million adults of 40-70 years old in the United States. The cataractous blindness rate is much privileged in African countries. This rate is as low as 5% in USA, Australia and UK while as high as 55% in some African countries to 58.5% higher in a few other countries[1]. In developing countries like Pakistan, the blindness rate is much higher while vision disability cases due to cataract are above 90% throughout the developing world[2]. Hence the senile cataract is the leading cause of human blindness over the world. It is actually an opacity that forms in the center of the aging ocular lens and is the major cause of this disease[3].

Cataract is linked with protein miss-folding and protein aggregation within the lens. Pathological analysis of cataractous lens nuclei revealed that, during aging, under certain conditions (temperature, heat, radiations etc.) several post translational modifications occur in crystallins that cause aggregation, cross linking, water insolubilization and lead to the formation of cataract by blocking the passage of light that reach the photoreceptors of retina[4, 5]. These post translational modifications that affect the lens opacity usually involve disulfide bonding, Lysine modification by carbamylation, Glycation, Cross-links formation relating lysine and arginines, Oxidation by UV light, formation of free radicals and a mixture of degradation products[6-8].

In vertebrate lenses, crystallin is the main protein class as the major portion of the lens protein is crystallin which accounts more than 90% of the total lens protein[9]. Mammalian lenses contain three types of this crystallin protein. These three types of structural protein are known as α, β and γ-crystallins. Among these, α and β-crystallins found as oligomers while γ-crystallin is exist as a monomer. These crystallins contribute to the transparency of the lens due to their special structural interactions and high concentrations[5]. In some cases, α-crystallins are seen as multimeric

assemblies that have chaperon like function. Over the previous decade, it has been broadly studied that even a single amino acid change in crystallin can show the way to formation of cataract. Fascinatingly these minor changes are usually seen in primary structure of proteins only. However smaller changes can be perceived in secondary or tertiary structure. α-crystallin, inspite of chaperon like function, also plays a crucial role in avoiding protein aggregation[10].

The powerhouse of proteomics is 2-D gel-electrophoresis and mass spectrometry which allows one to separate several thousands of proteins. The major drawback of this approach is that the separation is time consuming and laborious and it cannot be automated[11]. An alternative approach is reverse phase HPLC and GPC. HPLC column was successfully applied in protein purification and isolation techniques. The mobile phase as well as the stationary phase in HPLC has the potential to induce, stabilize or alter the configuration of proteins. The diagnostic tool HPLC provides retention information on the conformation and biophysical properties of proteins. It must be emphasized that conformational states of proteins are often sensitive to subtle changes in mobile phase composition and column temperature[12].

Aqueous chromatography based on gel filtration has also become popular since the introduction of cross-linked dextran supports in 1959, where fractionation is dependent primarily on differences in molecular size[13]. Subsequent characterization of human lens proteins on the basis of size and charge under denaturing or native conditions can be performed by several techniques. Size characterization can be best achieved by using gel permeation chromatography. Analysis by gel permeation chromatography showed that the soluble human cataractous eye lens proteins are usually size-fractionated into two crystallin groups, sometimes the peak doubling for two closely related isomers of crystallin. Determination of subunit composition and their molecular weights were conducted by SDS-PAGE[14].

The objective of this study was to identify and characterize crystallin classes in human cataractous eye lens nucleus. This study presents an investigation of protein distribution patterns across the human cataractous lens nucleus. The results of the current study also demonstrated the wide variety of crystallins in lens nucleus sample. For further identification, the subunit compositions of the peak fractions were determined by SDS-PAGE. Results also describe an analysis of the banding patterns of total soluble and insoluble proteins extracted from lens.

MATERIALS AND METHODS

Sample Collection

Twenty two fresh human cataractous lenses were procured from patients of different age group (40-80 years) with different etiologies, were obtained from different eye hospitals. These lenses were fractionated into four concentric fractions by controlled dissolution in phosphate buffer. Proportions of soluble and insoluble protein were determined by ninhydrin assay. Size exclusion fractions were separated by reverse phase HPLC.

Chemicals Required

All chemicals and reagents were purchased from Sigma-Aldrich, Merck and Acros Organics (above 98% purity) and were used without additional purification. Trichloroacetic acid (TCA), Thiourea, 2-mercaptoethanol, Sodium hydroxide (NaOH), Sodium dihydrogen phosphate (NaH_2PO_4), Sodium hydrogen phosphate (Na_2HPO_4) and Sodium chloride (NaCl). Methanol was of HPLC grade. All other chemicals were used as such without any further purification.

Sample Preparation

The human eye lens nucleus extracts were prepared as described by Truscott and Augusteyn[15]. Lens nucleus proteins were separated into four fractions on the basis of their

solubility in different media. These fractions were buffer soluble proteins (PS), urea soluble proteins (PU), yellow fraction proteins (PY) and insoluble proteins (PI).

Estimation of Protein Fractions

All four protein fractions were estimated by using different qualitative and quantitative methods. The protein fractions were qualitatively analyzed by Ninhydrin and Biuret method while spectrophotometric analysis was used as quantitatively to analyze soluble fractions using Bovine Serum Albumin (BSA) as standard. Absorbance was taken at 280 nm.

Methods of Analysis

Two methods are adopted for the analysis of protein pattern of human cataractous eye lens nucleus.

High Performance Liquid Chromatography (HPLC)

Reverse phase high performance liquid chromatography (RP-HPLC) differentiates partially purified proteins according to their hydrophobicity. All three soluble protein fractions, PS, PU and PY, were studied by chromatography on a 4.6 × 250 mm column C_{18} (Shim-Pack) equilibrated with 0.02 M sodium-phosphate buffer at pH 6.0 in presence of 0.006 M NaCl. Stationary phase consists of macroporous crosslinked hydrophilic polymer and the mobile phase was sodium phosphate buffer. Organic solvents and the reagents can also be used as mobile phase. Peaks were observed at a flow rate of 1.0 ml min^{-1}. Peaks were identified at 280nm using UV-Visible detector.

Gel Permeation Chromatography (GPC)

Gel permeation chromatography (GPC), based on size exclusion, was carried out at room temperature in prepacked column containing PL Aqua Gel. The mobile phase composed of 0.02 M sodium-phosphate buffer (Na_2HPO_4-NaH_2PO_4) at pH 6.9. The mobile phase was passed through

a Millipore filter having pore size 0.45 µm and degassed under vacuum before use. Sample was passed through superpure filter having pore size 0.22 µm. Elution was performed at a constant flow rate of 1.0 ml min^{-1}. Protein detection was done with a differential refractometer.

RESULTS

Human cataractous eye lens nucleus proteins were subjected to HPLC and GPC using C_{18} and PL aqua gel columns respectively. Fractions of crystallin with hydrophobicity were obtained through HPLC using C_{18} column while fractions of crystallin with molecular weights ranging from 12 KDa to 70 KDa were obtained through GPC using PL aqua gel column. The elution patterns for both, HPLC and GPC, are shown in Fig. 1-3. For identification, the molecular weights of the fraction peaks were determined using differential refractometer in case of GPC. All fractions were eluting between 10 and 15 minutes. Studies of water-insoluble crystallin using SDS-PAGE have demonstrated extreme homogeneity with evidence of major components with molecular masses of 18-43 KDa, similar to the crystallin of water-soluble portion[8] as shown in Fig 4.

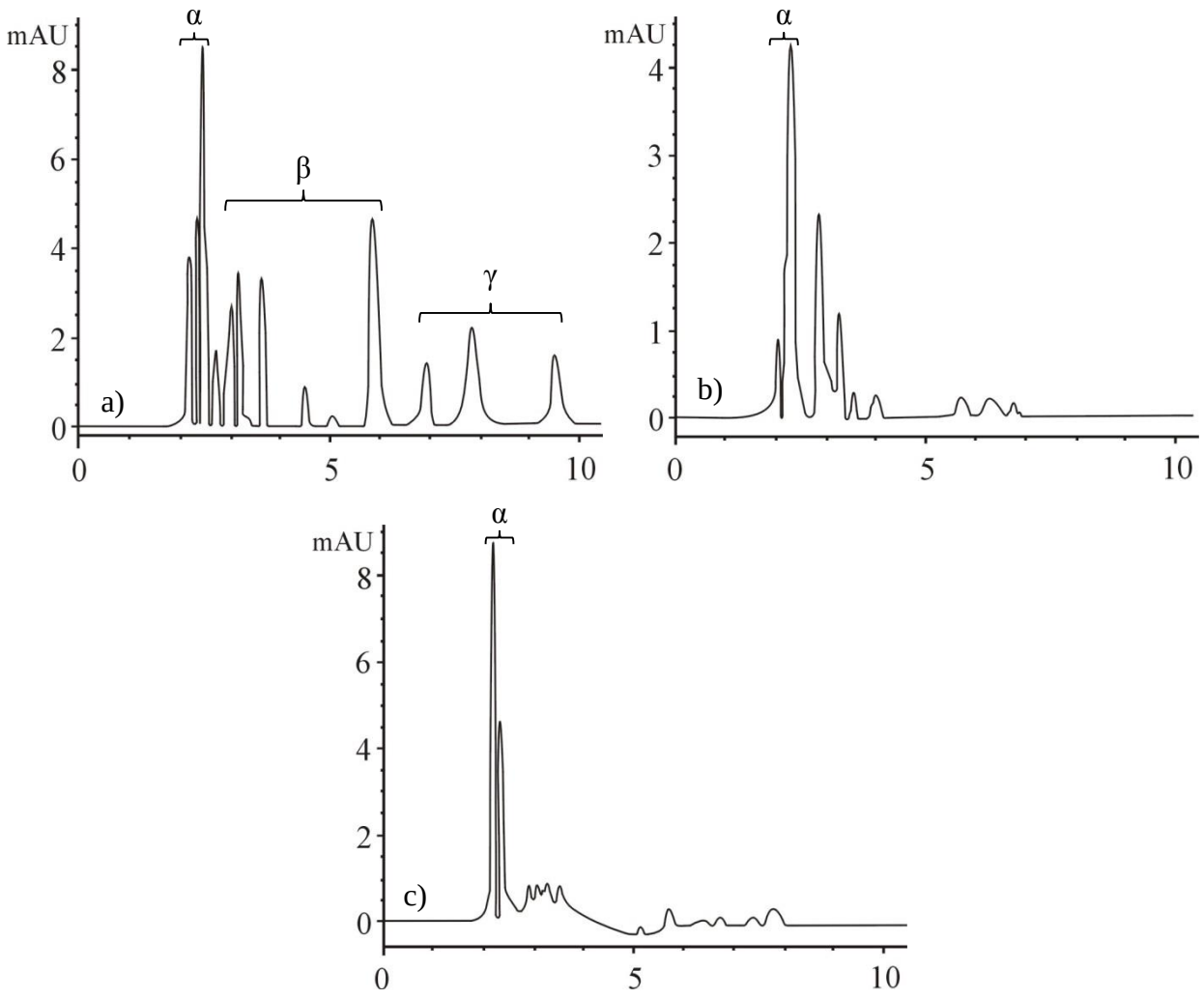

Figure 1: HPLC analysis; a) Buffer soluble fraction (PS), b) Urea soluble fraction (PU) and c) Yellow fraction (PY) of Human Cataractous Eye Lens Nucleus Protein.

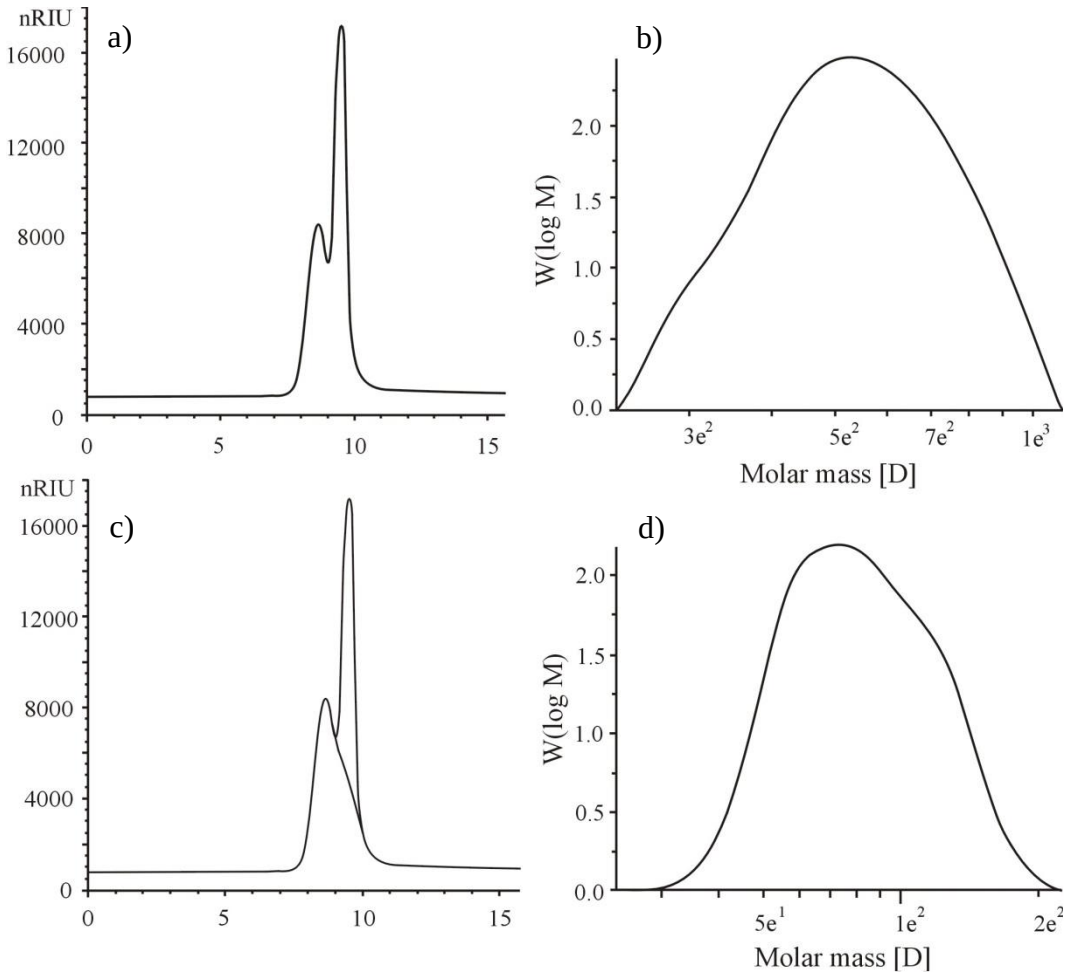

Figure 2: GPC analysis; a, c) Buffer soluble fraction (PS), b, d) Molar mass distribution curve of protein sample (PS) of Human Cataractous Eye Lens Nucleus Protein.

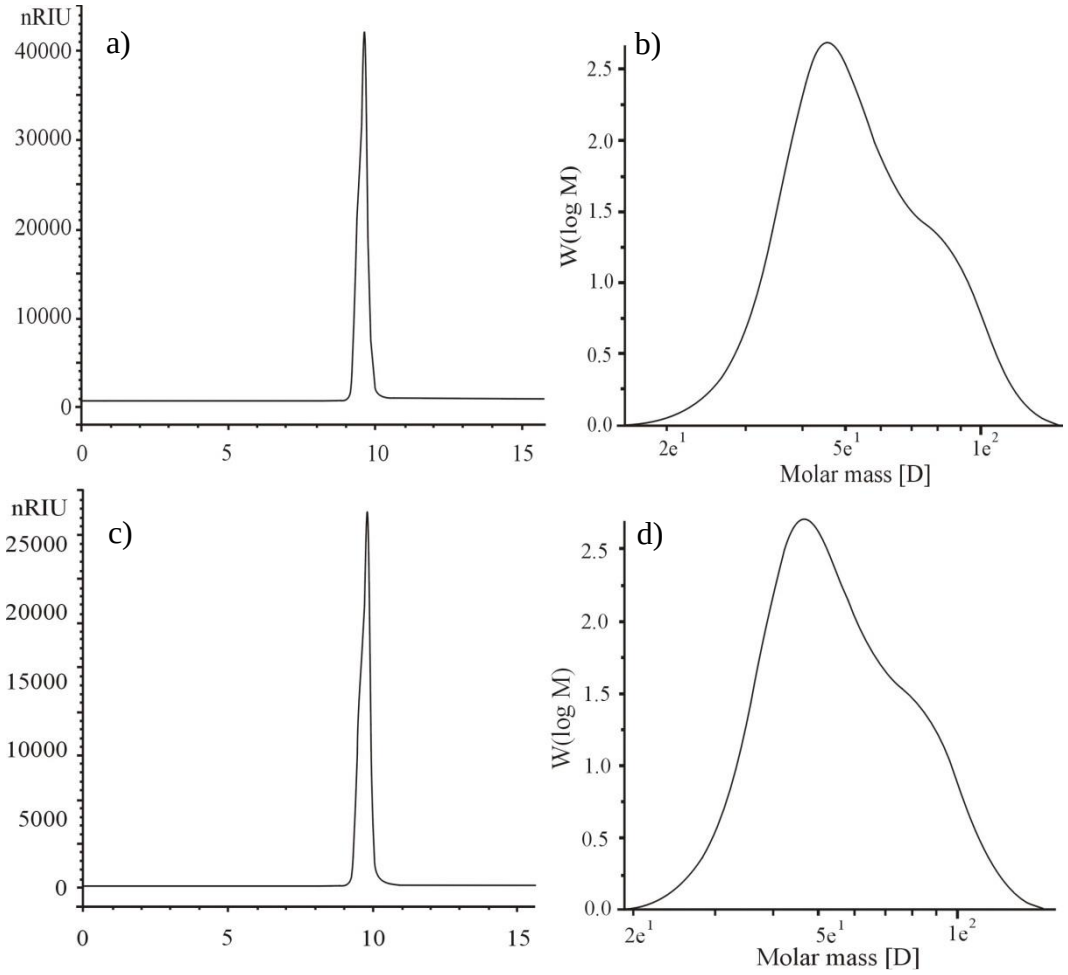

Figure 3: GPC analysis; a) Urea soluble fraction (PU) and b) Molar mass distribution curve of protein sample (PU), GPC analysis; c) Yellow fraction (PY) and d) Molar mass distribution curve of protein sample (PY) of Human Cataractous Eye Lens Nucleus Protein.

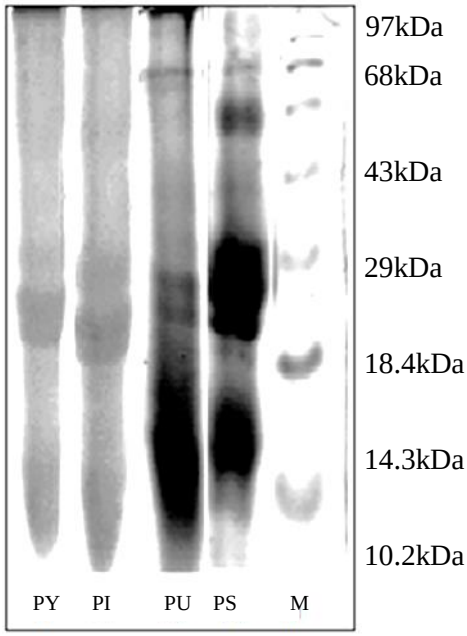

Figure 4: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) of the soluble and insoluble proteins extracted from the nuclear region of human cataractous eye lens. PY represents Yellow fraction protein, PI stands for Insoluble protein fraction, PU symbolizes Urea soluble protein fraction, PS corresponds to Buffer soluble protein fraction while M is the marker protein.

DISCUSSION

It is shown that HPLC with C_{18} column and GPC with PL aqua gel column are excellent methods for analytical and semi-preparative fractionation of lens nucleus proteins. Molecular weights determination, on the basis of hydrophobicity, was performed using a photometer by taking absorbance at 280 nm[12]. On the basis of size, the molecular weights determination was performed using a differential refractometer[14].

HPLC analysis of Buffer soluble protein fraction (PS) shows that there are various proteins present in this fraction as it shows more than one peak in Fig 1(a). The chromatogram shows well resolved peaks of crystallin protein isomers α, β and γ, actually the group of peaks mark as α is comprises of itself α and its sub groups αA, αB. The retention time of this group is less than three minutes. Similarly in buffer soluble fraction the second group of peaks is of β crystallin protein

having various peaks corresponding to βA, βB, and even their further sub groups like βA3, βA4, βB1 and βB2. Third group of peaks in buffer soluble fraction comprises of γ crystallin protein showing three peaks clearly indicating the sub groups γC, γD and γS.

The pattern of peaks in human eye cataractus lens nucleus sample (PS) is the similar obtained in soluble fractions using Bovine Serum Albumin (BSA) as standard. All components present in buffer soluble fractions was eluted out within 15 minutes by using a simple Isocreatic elution method with UV detection, commonly used detection technique and almost available in every scientific/diagnostic laboratory. The newly developed HPLC method was found to be suitable for the analysis of various crystallin proteins in human eye cataractus lens nucleus. The method was validated as per International Conference Harmonization guidelines for various parameters like accuracy, precision, linearity, robustness, system suitability. The results obtained in HPLC analysis of PS fraction are supporting to the results already reported in literature. According to reported literature[5] these various proteins may be crystallin protein isomers i.e α, β and γ having closely related masses. These, β and γ are further classified as αA, αB, βA, βB and γC, γD and γS. These are even further sub grouped as βA3, βA4, βB1 and βB2. Literature also reveals that αA, αB crystallin are a major protein component of the mammalian eye lens.

In GPC analysis only two peaks were obtained as compared to many peaks in HPLC. These results clearly indicate that on the basis of size exclusion our sample contains various isomers of crystallin protein having two sets of mass. The weight average and number average molecular weights of peak 1, M_W and M_N, amount to 5.5781×10^2 and 5.0069×10^2, respectively revealing a value of 1.1141 for polydispersity factor, M_W/M_N, also denoted as D. For the peak 2, the weight average amount to 8.6071×10^1 and number average molecular weights amount to 7.5379×10^1, revealing a value of 1.1418 for polydispersity factor. The molar mass distribution parameters for

the various isomers of soluble fraction are given in table 1. All above discussed isomers can be divided in to two groups according to their molecular size.

Table 1: Molar Mass Distribution Parameters for Soluble Fractions of Crystallin Protein.

Parameters	**Buffer Soluble Fraction (PS)**		**Urea Soluble Fraction (PU)**	**Yellow Fraction (PY)**
	Peak 1	**Peak 2**		
M_W	5.5781×10^{2}	8.6071×10^{1}	5.6256×10^{1}	5.8416×10^{1}
M_N	5.0069×10^{2}	7.5379×10^{1}	4.9415×10^{1}	5.1687×10^{1}
D	1.1141	1.1418	1.1384	1.1302
M_Z	6.1680×10^{2}	9.8106×10^{1}	6.4194×10^{1}	6.6173×10^{1}
V_P	8.6327	9.5183	9.7343	9.7198
M_P	5.2550×10^{2}	7.3444×10^{1}	4.5450×10^{1}	4.6938×10^{1}
A	9.0108×10^{2}	5.9809×10^{3}	1.6054×10^{5}	1.0153×10^{5}

In HPLC analysis of Urea soluble fraction (PU), two sets of peaks are shown as given in Fig 1b. Both these set of peaks are not much away from each other presenting that these have some resemblance among each other. The similar pattern of peaks was obtained upon the analysis of Bovine Serum Albumin (BSA) as standard in PU fraction. The numbers of peaks are lesser in both set of peaks corresponding to α, β in PU fraction as compared to PS fraction.

It was cleared from GPC analysis as only one peak is obtained in GPC analysis of Urea soluble fraction showing that both isomers of crystallin protein is present in this part having similar molar mass distribution. The weight average (M_W) and number average (M_N) molecular weights of this single peak have values 5.6256×10^{1} and 4.9415×10^{1}, respectively illuminating the amount of 1.1384 for polydispersity factor (D).

Yellow fraction (PY) protein upon HPLC analysis also gives an idea about one set of peaks adjacent to each other as shown in Fig 1c, similarly obtained with standard. In this analysis the retention time of peaks are corresponding to α crystalline protein. All other components of crystalline protein are not present in PY fraction. In GPC analysis of Yellow fraction portion, only one peak is observed as in Urea soluble fraction. This also indicates that only one type of isomers is present in this fraction on the basis of size exclusion. For this only peak, the M_W and M_N molecular weights amount to 5.8416×10^1 and 5.1687×10^1, respectively revealing 1.1302 value for polydispersity factor.

SDS Polyacrylamide gel electrophoresis (Fig. 4) of different protein fraction such as PY, PI, PU and PS along with protein marker gave different number of bands in each case as depicted earlier by Roy and Spector in 1976[16]. Both insoluble and soluble protein fractions gave almost similar bands among each other. Based on standard polypeptide markers, molecular weights ranging from 14.5 KDa to 70 KDa were found in each fraction. It seems that 20 KDa fraction appears to be a major component in all protein fractions.

According to the known sizes of polypeptides of the lens protein, it is probable that 18 KDa to 22 KDa polypeptide is α-crystallin while β-crystallin constitutes to 29 KDa to 43 KDa. It is assumed that 14 KDa may be γ-crystallin[17].

It has been shown in Fig. 1a) that PS shows various bands as many peaks of this fraction were seen in HPLC analysis while only two peaks were depicted by GPC results showing that on the basis of molar masses, components were fall in two molecular weight categories. PU fraction is showing three bands as in HPLC analysis only one peak of this fraction was appeared. PY and PI fractions show only two bands. HPLC and GPC analysis also supports these results as only one peak was appeared in case of PY. We are reporting this study first time, using HPLC for the

analysis of three different fractions of crystallin protein extracted from human eye cataractus lens. HPLC analysis was carried out by developing simple, isocratic, reproducible and robust method along with developing a GPC method for the molar mass distribution determination. The results obtained by HPLC method was correlated with GPC analysis.

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