

# Differential proteomics analysis of proteins from human diabetic and age-related cataractous lenses

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**Background:** To investigate the differential lens proteomics between diabetic cataract, age-related cataract, and natural subjects. **Materials and Methods:** Two-dimensional electrophoresis (2-DE), mass spectrometry (MS), and enzyme-linked immunosorbent assay (ELISA) were employed. Total soluble proteins in lenses of type I diabetic cataract, age-related cataract (nondiabetic) patients, and normal control were extracted and subjected to 2-DE. The differential protein spots were recovered, digested with trypsin, and further applied to MALDI-TOF-MS. ELISA analysis was used to determine the levels of differential proteins in lenses of three groups. **Results:** 2-DE analysis reflected that lens proteins of normal control, diabetic, and age-related cataract subjects were in the section of pH 5-9 and the relative molecular weights were 14-97 kDa, while relative molecular weight of more abundant crystallines was localized at 20-31 kDa. five differential protein spots were detected and identified using MALDI-TOF-MS, including beta-crystallin A3, alpha-crystallin B chain, chain A of crystal structure of truncated human beta-B1-crystallin, beta-crystallin B1, and an interesting unnamed protein product highly similar to alpha-crystallin B chain, respectively. ELISA analysis revealed that lenses of diabetic cataract patients should contain significantly more concentrations of beta-crystallin A3, alpha-crystallin B chain, and beta-crystallin B1 than those of age-related cataract patients and normal control. **Conclusion:** This study clearly reflected the differential proteins of diabetic cataract, age-related cataract lenses compared with natural subjects, and it is helpful for the further research on the principles and mechanisms of different types of cataract.

**Key words:** Age-related cataract, crystalline, diabetic cataract, mass spectrometry, proteomics, two dimensional electrophoresis

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## INTRODUCTION

Cataract is considered as a major cause of visual impairment in diabetic patients, as the incidence and progression of cataract is increasingly elevated in patients with diabetes mellitus.<sup>[1,2]</sup>

Recently, cataract surgery is an effective and the most common surgical ophthalmic procedure worldwide. However, the elucidation of pathomechanisms to delay or prevent the development of cataract in diabetic patients remains a challenge. Furthermore, patients with diabetes mellitus suffer higher complication proportions from cataract surgery.<sup>[3]</sup> Both diabetes and cataract pose an enormous health and economic burden, particularly in developing countries, where diabetes treatment is insufficient and cataract surgery often inaccessible.<sup>[4]</sup>

The transparency and stability of the eye lens is achieved by tight packing of crystallin proteins into a glass-like microarchitecture.<sup>[5]</sup> The crystallins comprise 80%-90% of the soluble lens protein mass and are divided into three basic types termed  $\alpha$ ,  $\beta$ , and  $\gamma$ .<sup>[5]</sup> Crystallin proteins located in the central and equatorial regions of the lens are normally stable and do not turnover, since these cells

lose their nuclei during the developmental transition from epithelial to fiber cells.<sup>[6]</sup>

It was previously reported that the development of diabetes cataract should be associated with polyol pathway,<sup>[7]</sup> osmotic stress caused by sorbitol accumulation,<sup>[8]</sup> and free radical.<sup>[9]</sup> However, few researches reported differential proteomic analysis of proteins from diabetic cataractous human lenses.

In this study, crystallin proteins in the diabetic and age-related cataractous lenses were extracted and compared with those of normal subjects, using two-dimensional electrophoresis (2-DE), MALDI-TOF-mass spectrometry (MS), and enzyme-linked immunosorbent assay (ELISA) analysis. This was carried out to gain further insight into the significant observation of differences of age-related and diabetic cataract.

## MATERIALS AND METHODS

### Substrates and chemicals

The lenses were obtained from 20 age-related cataract patients ( $n = 20$ ,  $65.5 \pm 6.7$  years), 20 type I diabetic cataract patients ( $n = 20$ ,  $35.5 \pm 7.5$  years) and five normal

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controls (donated for corneal transplant in accordance with the Standardized Rules for Development and Applications of Organ Transplants and obtained from the Eye Bank of Shanghai, China.). Informed consent was obtained from all patients. The cataractous lenses were carefully examined by ophthalmologists prior to surgical removal and were all detected with nuclear cataract. Lenses of age-related and diabetic cataract were detected with grade III and grade II of nuclear cataract, respectively.

The prestained and unstained molecular weight protein markers were from Invitrogen (Carlsbad, CA, USA) and Amersham Biosciences (Piscataway, NJ, USA), respectively. All chemicals for two-dimensional (2D) gel electrophoresis were from either Amersham Biosciences or Bio Rad (Hercules, CA, USA). Other chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) or Fisher (Atlanta, GA, USA).

Monoclonal antibodies against beta-crystallin A3, alpha-crystallin B chain, and beta-crystallin B1 were purchased from Sigma.

#### The extraction of lens soluble proteins

Phacoemulsification was used to remove cortical region and was followed by irrigation and aspiration to remove the nuclear region. The recovered lenses should contain 10% of the original cortex and 90% of the nucleus. All samples were collected and stored at -80°C before use.

Each lens sample (~20 mg) was mixed with 1 mL phosphate buffered saline (20 mM, pH 7.2), disrupted by sonication, and then centrifuged at 10,000 g for 30 min at 4°C. To deplete high content of hyaluronic, which could severely interfere the 2-DE process, the supernatant was collected and incubated with 25% prechilled trifluoroacetic acid at 4°C for 2 h. The result solution was then centrifuged at 10,000 g for 10 min at 4°C. The pellet was washed with prechilled acetone for three times and centrifuged at 10,000 g for 10 min at 4°C and then dried in vacuum.

#### Two-dimensional gel electrophoresis analysis

Then the resulting pellets were dissolved in resolubilization buffer (5 M urea, 2M thiourea, 2% 3-[(3-cholamidopropyl)-dimethyl-ammonio-1-propane sulfonate] (CHAPS), 2% caprylyl sulfo betaine 3-10, 2 mM tri-butyl phosphine, 40 mM Tris, pH 8.0) and incubated with Immobiline Dry Strips (pH range of 3-10, Amersham Biosciences) overnight at 25°C. Each preparation was subjected to 2D gel electrophoresis (IEF in the first dimension followed by SDS-PAGE in the second dimension) by exactly following the manufacturer's handbook (Amersham Biosciences).

Following the IEF separation, the second dimension SDS-PAGE was performed with the Laemmli<sup>[10]</sup> method using a 15% polyacrylamide gel of 16 × 14 cm (width × height). After the first dimensional IEF separation, the strips were consecutively treated for 15 min each, first with 100 mM dithiothreitol (in equilibration buffer: 0.1 M Tris, pH 6.8, containing 6 M urea, 30% glycerol, and 1% SDS), and next with 300 mM iodoacetamide (also dissolved in the equilibration buffer). The protein spots on gel were stained with Coomassie blue.

#### MS analysis

25 μg trypsin was dissolved in 2.5 mL tosylphenylalanylchloromethane mixed with 250 μL of 0.1% redistilled acetonitrile. A 15 μL sample of this trypsin solution was activated in 100 μL 50 mM NH<sub>4</sub>HCO<sub>3</sub>.

Protein spots of interest were excised from gels and then transferred to microcentrifuge tubes. After washed with dH<sub>2</sub>O for two times, the gel spots were then washed with 200 μL of 25 mM ammonium bicarbonate for three times, followed by dehydrated with ACN. After dried down in a vacuum centrifuge, the gel-pieces were incubated in the activated trypsin solution for 16 h at 37°C. The resulting peptides were extracted three times by 20 mL aliquots of 5% TFA in 50% ACN. The extracts were pooled and then dried in a vacuum centrifuge.

MALDI-TOF-MS analysis was performed using AutoFlex MALDI-TOF-MS (Bruker, Germany). The resulting peptides were dissolved in 0.5% TFA and 5% ACN and mixed with matrix (saturated sinapinic acid in acetonitrile/H<sub>2</sub>O, containing 0.1% TFA, 1:1, v/v) to promote desorption and ionisation. An N<sub>2</sub> laser at 337 nm was used to desorb the solute molecules from the sample disc and a voltage of 20 kV were established in the source region. Data were analyzed by flex analysis (Bruker Daltonics). The results were subsequently investigated with the Matrix Science web site (<http://www.matrixscience.com/>) using "Mascot Search→Peptide Mass Fingerprint" to search for homologous proteins in the NCBI nr (National Center for Biotechnology Information nonredundant) database with the taxonomy set to "Homo sapiens."

#### ELISA analysis

To detect the concentrations of beta-crystallin A3, alpha-crystallin B chain, and beta-crystallin B1 in the soluble protein samples from lenses of normal control, diabetic, and age-related cataract patients, the extracted proteins were applied to ELISA analysis according to the method previously reported.<sup>[11]</sup> Monoclonal antibodies were first fixed into the plates and reacted with extracted lens proteins and then HRP-antibodies. Finally, the mixtures were reacted with tetramethylbenzidine substrate, after termination with

2M H<sub>2</sub>SO<sub>4</sub>, OD450 nm was detected for each well using a microtiter plate reader within 30 min.

## RESULTS

### 2-DE analysis

The soluble proteins extracted from the lenses of age-related cataract patients, type I diabetic cataract patients, and normal controls were subjected to 2-DE analysis.

The 2-DE gels [Figure 1] showed that most of the soluble proteins of control, diabetic cataract, and age-related cataract lenses were located in the section of pH 5-9 with the relative molecular weight at 14,000-97,000 Da, while relative molecular weights of more abundant crystallines were localized at 20,000-31,000 Da.

In control, diabetic cataract and age-related cataract groups, 73, 76, and 68 protein spots were detected, respectively. A total of five significant differential protein spots were selected by the PDQuest software [Figure 1].

The 2-DE analysis revealed that the type I diabetic cataract lenses contained the highest concentrations of proteins in spot 1, 2, 3, and 4, while control lenses contained the lowest contents of these proteins. Additionally, age-related cataract lenses were detected with the highest concentration of the protein in spot 5.

### MALDI-TOF-MS analysis

A total of five selected protein spots were extracted from the gels, hydrolyzed with trypsin, and applied to MS analysis. The resulting MS data were investigated with the Matrix Science online service to search for homologous proteins.

Peptides of five crystallin and related proteins were detected: The protein in spot 1 was hydrolyzed into 16 peptides and 7 peptides were identical with segments of beta-crystallin A3, with the matched sequence coverage at

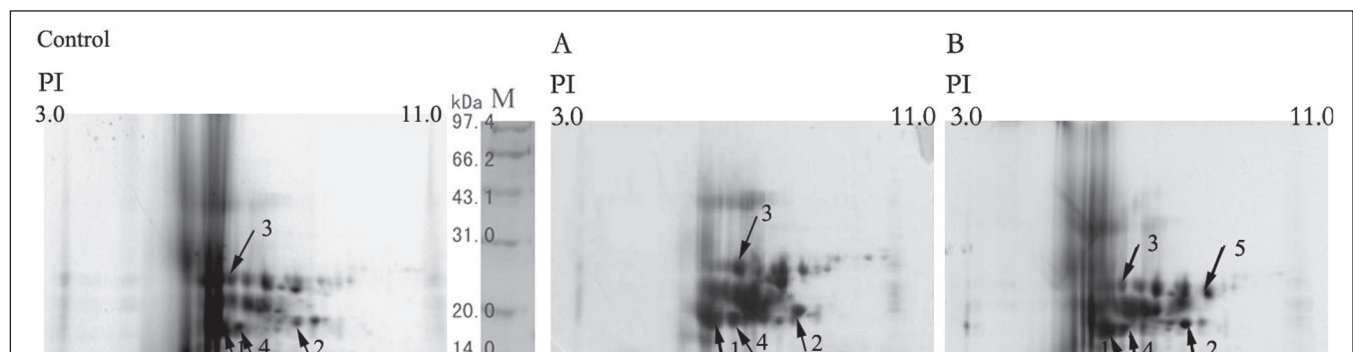
40% [Figure 2]; the protein in spot 2 was hydrolyzed into 26 peptides, and 6 ones were identical with segments of alpha-crystallin B chain, with the matched sequence coverage at 45% [Figure 3]; the protein in spot 3 was hydrolyzed into 10 peptides, and 7 ones were identical with segments of chain A, crystal structure of truncated human beta-B1-crystallin, with the matched sequence coverage at 49% [Figure 4]; the protein in spot 4 was hydrolyzed into 25 peptides, and 6 ones were identical with segments of beta-crystallin B1, with the matched sequence coverage at 34% [Figure 5]; and the protein in spot 5 was hydrolyzed into 11 peptides, and 5 ones were identical with segments of an interesting unnamed protein product, which is highly similar to alpha-crystallin B chain, containing an alpha-crystallin A chain region at N terminal, and an alpha-crystallin-Hsps\_p23-like domain, the matched sequence coverage was 49% [Figure 6].

### ELISA analysis

In ELISA analysis, the concentrations of beta-crystallin A3, alpha-crystallin B chain, and alpha-crystallin chain A in the three protein samples from lenses of control, diabetic, and age-related cataract patients were determined. The results revealed that type I diabetic cataract lenses contained much more beta-crystallin A3, alpha-crystallin B chain, and beta-crystallin B1 than the other two groups; additionally, the concentrations of the three proteins in age-related cataract lenses were detected as a little higher than those of control subjects [Figure 7].

## DISCUSSION

Diabetes is a known risk factor for cataract formation. In view of the prevailing and predicted outbreak of diabetes in developing countries like India,<sup>[12,13]</sup> diabetic cataract may become a leading cause of blindness, along with age-related cataract. In the present study, we comparatively analyzed the separated protein spots on 2-DE gels of normal control, age-related, and diabetic cataract lenses. The whole lenses were extracted from age-related and diabetic cataract



**Figure 1:** Two-dimensional electrophoresis analysis of soluble proteins extracted from normal control, age-related cataractous, and diabetic cataractous lens. (Control) Soluble proteins extracted from normal control lenses. (a) Soluble proteins extracted from age-related cataractous lenses. (b) Soluble proteins extracted from diabetic cataractous lenses. Five protein spots with significantly differential concentrations were pointed with arrows and then recovered and subjected to mass spectrometry analysis

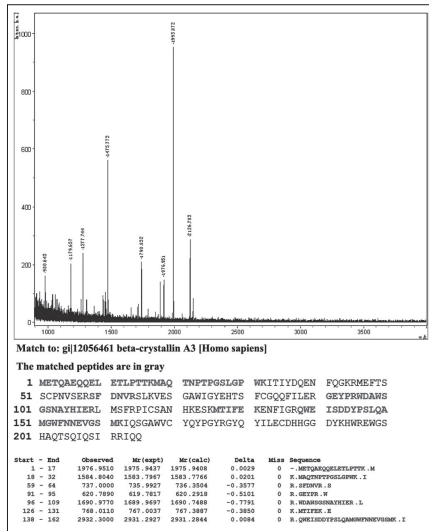


Figure 2: Mass spectrometry analysis of spot 1 The protein extracted from spot 1 was digested with trypsin and 16 peptides were detected. The protein was then identified as gi|12056461, beta-crystallin A3 with seven matched peptides

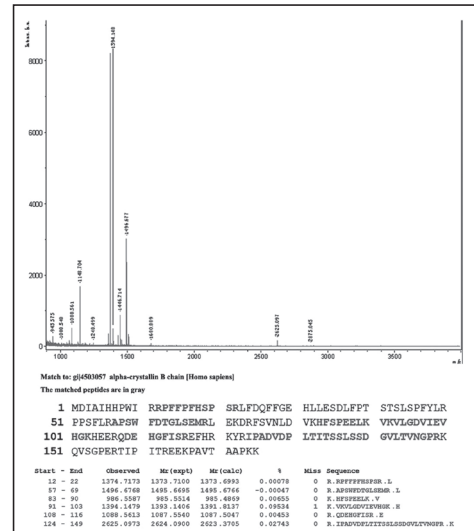


Figure 3: Mass spectrometry analysis of spot 2 The protein extracted from spot 2 was digested with trypsin and 26 peptides were detected. The protein was then identified as gi|4503057, alpha-crystallin B chain with six matched peptides

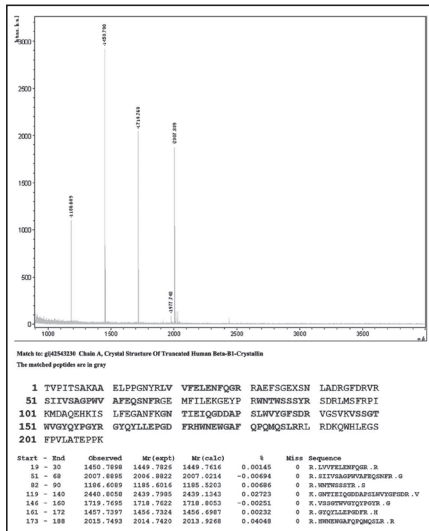


Figure 4: Mass spectrometry analysis of spot 3 The protein extracted from spot 3 was digested with trypsin and 10 peptides were detected. The protein was then identified as gi|42543230, crystal structure of truncated human beta-B1-crystallin with seven matched peptides

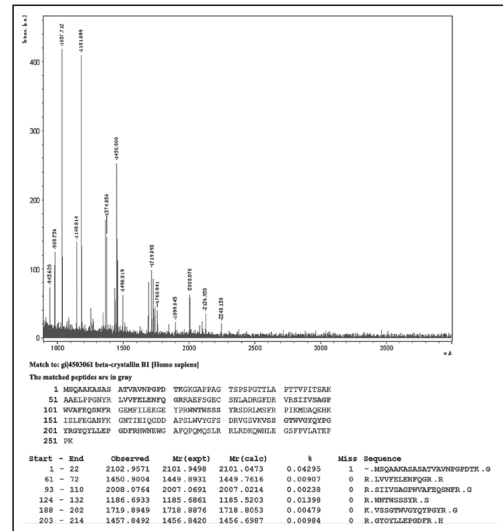


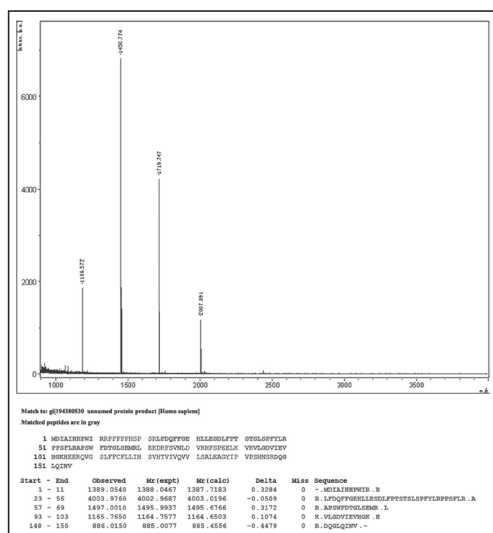
Figure 5: Mass spectrometry analysis of Spot 4 The protein extracted from spot 4 was digested with trypsin and 25 peptides were detected. The protein was then identified as gi|4503061 beta-crystallin B1 with six matched peptides

patients. No distinction was found in this study regarding changes between the cortical and nuclear regions of these lenses.

In 2-DE and MS analysis, five significantly differential protein spots were detected, including beta-crystallin A3, alpha-crystallin B chain, chain A of crystal structure of truncated human beta-B1-crystallin, beta-crystallin B1, and an unnamed protein product. The gels revealed that type I diabetic cataract lenses should contain much more beta-crystallin A3, alpha-crystallin B chain, chain A, crystal structure of truncated human beta-B1-crystallin and beta-crystallin B1 than the other two groups. And in ELISA analysis, type I diabetic cataract lenses were also proved to

contain more contents of beta-crystallin A3, alpha-crystallin B chain, and beta-crystallin B1 than age-related cataract and control lenses [Figure 7].

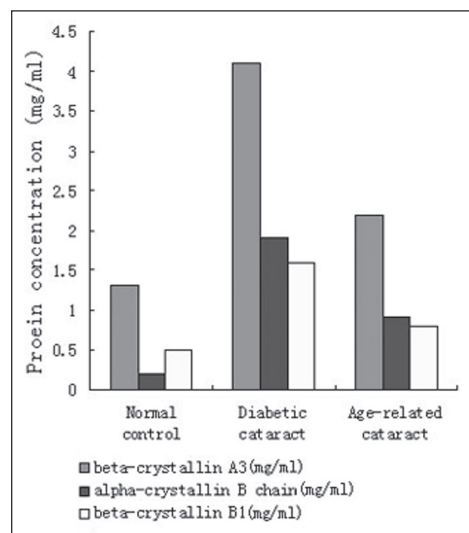
It was interesting that a significant protein spot was detected in age-related cataract lenses; it was not entitled and could only be identified from a cDNA sequence cloned from human hippocampus. It was highly similar to alpha-crystallin B chain, containing an alpha crystallin A chain region at N terminal, and an alpha-crystallin-Hsps\_p23-like domain, seldom mentioned in cataract, and its role and principle in cataract were still unclear. However, this protein might be helpful in the further research on the mechanism of age-related cataract.



**Figure 6:** Mass spectrometry analysis of spot 5 The protein extracted from spot 5 was digested with trypsin and 11 peptides were detected. The protein was then identified as gi|194380530, unnamed protein product with five matched peptides

As previously reported, beta-crystallin oligomers were suggested to play a critical role in maintenance of lens transparency<sup>[14]</sup>; and the truncation of beta-B1-crystallin was associated with its structure and stability.<sup>[15-17]</sup> And diabetic may enhance NH<sub>2</sub>- and COOH-terminal extensions of beta-B1-crystalline, which could accelerate lens opacity and oligomer formation.<sup>[18]</sup> In our study, higher contents of beta-B1-crystalline and truncated human beta-B1-crystallin were determined in diabetic cataract lenses, and it was consistent with early reports.

Alpha-crystallin was reported to constitute the major portion of eye lens cytoplasm and its concentration in the lens could reach up to 50% of the total proteins; it displayed chaperone-like activity in suppressing the aggregation of various proteins and in preventing inactivation of enzymes due to heat and other stress conditions. Alpha-crystallin, especially alpha-crystallin B, was also known to undergo extensive posttranslational modifications including oxidation, mixed disulfide formation, truncation, and glycation during aging,<sup>[19]</sup> and was believed to be the key structural and functional element for maintaining the transparency of the lens. Furthermore, it was known that the chaperone activity of  $\alpha$ -crystallin is compromised in various types of cataract, including diabetic cataract.<sup>[10,20-22]</sup> It was reported that alpha-crystallin from diabetic rat and human lenses had shown a substantial loss in their chaperone function,<sup>[23,24]</sup> in addition,  $\alpha$ -crystallin chaperone activity was also found to be impaired in galactosemic rat lenses.<sup>[25]</sup> Owing to the loss of its chaperone function, more alpha-crystallin should be expressed by the surround cells and tissues; therefore, in this research, significantly higher alpha-crystallin B chain concentration could be detected in type I diabetic lenses.



**Figure 7:** Enzyme-linked immunosorbent assay analysis The protein concentrations of beta-crystallin A3, alpha-crystallin B chain, and beta-crystallin B1 were determined in lenses of normal control, type I diabetic cataract and age-related cataract patients

Additionally, the contents of beta-crystallin A3 and was also studied in age-related cataracts lenses.<sup>[26]</sup>

## CONCLUSION

In this study, proteins extracted from control, type I diabetic, and age-related cataracts lenses were applied to 2-DE, MS, and ELISA analysis. Type I diabetic cataract lenses should contain the highest contents of beta-crystallin A3, alpha-crystallin B chain, beta-crystallin B1, and chain A of crystal structure of truncated human beta-B1-crystallin. Compared with control subjects, age-related cataracts lenses revealed slightly higher concentrations of the four proteins above; additionally, gi|194380530, an unnamed crystalline-like protein was also detected in this group with significantly higher content.

Furthermore, the identification of these differential proteins could help to diagnose the certain types and grades of nuclear cataract in clinical research and treatment, and could also bring benefits in the further research on the principle of cataract. The lens samples could only be extracted and studied through surgery after cataract was formed and detected; therefore, to reveal the pathogenesis in the early stage of cataract, more attention could be devoted to gene research. To monitor the expression levels of the genes for these differential proteins should be of great potential in the further research of the mechanism of cataract.

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