Veterinary Ophthalmology (2015) **18**, 2, 109–115 DOI:10.1111/vop.12113

Serum antibodies against βH-crystallins in the American Cocker Spaniel

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Abstract

Objective To detect antibodies for lens βH-crystallins in the serum from the American Cocker Spaniel (ACS) presenting with and without cataracts and with and without uveitis.

Animal Studied Seventy-three American Cocker Spaniels and six normal Beagles.

Procedures Sera were collected from 73 ACSs, including those with normal lenses and those with cataracts, or uveitis. Fractionated, normal Beagle lens βH-crystallins were separated by one- or two-dimensional electrophoresis. The separated lens βH-crystallins were used on immunoblots as sentinel substrates against which the ACS sera were tested for the presence of antibodies against βH-crystallins.

Results Sera from approximately two-thirds of study animals contained antibodies to some βH-crystallin polypeptides, but reactivity varied among patients. Contrary to some hypotheses, serum antibodies to groups of βH-crystallins did not relate to the stages of cataract. However, detailed analysis by two-dimensional immunoblotting and mass spectrometry showed that three spots originating from βA1-crystallin were detected only in sera from cataract patients.

Conclusion Serum antibodies to βA1-crystallin may be associated with the development of cataract.

Key Words: American Cocker Spaniels, cataracts, dog, immunoblotting, serum antibodies, βH-crystallins

INTRODUCTION

Cataracts are a leading cause of poor visual acuity and blindness in dogs. A retrospective study reported significantly higher odds ratios for cataracts in six pure-bred dogs (including cocker spaniel, miniature schnauzer, toy poodle, Boston terrier, miniature poodle, and bichon frise) compared with mixed-breed dogs.[1] For example, the American Cocker Spaniel (ACS) was reported in North America to have a prevalence of cataract of 8.8% during the period of 1964–2003,[2] and similar prevalence (7.8%) was also reported in Brazil during the period of 2005–2008.[3] Risk factors for cataract include the following: congenital defects, advancing age, genetic background, diabetes mellitus, uveitis, hypocalcemia, electric shock, and exposure to radiation or toxic substances, such as dinitrophenol and naphthalene.[4,5]

Postulated mechanisms for cataract formation include the following: (i) action of reactive oxygen species leading to breakdown of lens plasma membranes, (ii) loss of ion homeostasis and accumulation of sodium and calcium in lens, and (iii) post-translational modifications of the major structural proteins of the lens (α-, β-, and γ-crystallins) leading to their insolubilization and opacity.[6–11] Post-developmental modifications, include truncation, phosphorylation, and deamidation. Another postulated mechanism is that the plasma membranes of the lens leak crystallins into the anterior chamber and systemic circulation, causing an autoimmune reaction to lens proteins, cataract formation, and uveitis.[12] As this latter mechanism is controversial, the purpose of this study was to determine the relationship between serum antibodies to βH-crystallins and the stage of cataract in ACS. We focused on the β-crystallins because they are one of the

most abundant components of the insolubilized, cataractous lens proteins.[13,14] Although nonlenticular tissues (e.g., retina) may express low levels, the lens contains the highest concentration of β-crystallins.[15,16]

MATERIALS AND METHODS

Animals

Seventy-three American Cocker Spaniels (40 males and 33 females) with medical records at the Veterinary Teaching Hospital of Azabu University during October 2003 to February 2010 were used. To collect normal lens proteins, eyes from six 2-year-old healthy Beagles (three males and three females) were enucleated in protocols not related to the present studies. Lenses were obtained by intracapsular surgery and then stored at −80 °C until use. All experimental animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23). The protocols were approved by the animal care and use committee of Azabu University.

As an inclusion criteria, all animals received a thorough ophthalmic examination including neuro-ophthalmic examination, Schirmer Tear Test (Schirmer Tear Test strips; Eagle Vision, Memphis, TN, USA), fluorescein dye staining (Fluores Ocular Examination Test Paper; Showa Yakuhin Kako Co., Ltd, Tokyo, Japan), applanation tonometry (Tonopen XL; Medtronic Solan, USA), slit-lamp biomicroscopy equipped with a CCD camera (Kowa SL-14; Kowa Co, Tokyo, Japan), and indirect ophthalmoscopy. Stages of cataract was classified as immature, mature, or hypermature cataracts according to the criteria reported by Leasure, *et al.*[17] Both eyes were scored for cataracts, and only data from the eye with the cataract in the most advanced stage were reported. Uveitis was also scored by observing conjunctival hyperemia, iris appearance and aqueous flare, as modified previously by Park, *et al.*[18] Score 0 was defined as no conjunctival hyperemia, no iris hyperplasia or atrophy, and no aqueous flare; score 1 was defined as mild conjunctival hyperemia, mild iris hyperplasia or atrophy, but no aqueous flare; score 2 was defined as moderate conjunctival hyperemia, moderate iris hyperplasia or atrophy (possible darkening of the iris), and no or mild aqueous flare; and score 3 was defined as severe conjunctival hyperemia, severe iris hyperplasia or atrophy, and moderate aqueous flare.

Isolation and purification of lens proteins

Frozen lenses obtained from six 2-year-old Beagles were homogenized, and total water-soluble lens proteins were collected after centrifugation. Lens crystallins were fractionated using Sepharose CL-6B gel filtration. Groups of βH-crystallins, termed H1, H2, H3, and H4, were further separated using 12.5% SDS-polyacrylamide gel electrophoresis using a Mini-PROTEAN® Tetra cell (Bio-Rad Laboratories, Inc., Tokyo, Japan). These βH-crystallins were used as sentinel proteins for detecting serum antibodies described in the following sections because of previous comparative literature data,[19] the known masses and migration positions of the seven separate gene products for β-crystallins polypeptides on electrophoresis, and the propensity of β-crystallins to become modified and precipitate in experimental cataract.[13,20]

Detection of serum antibody reactivity by immunoblotting

βH-crystallins separated by SDS-PAGE gels were electrotransferred to polyvinylidene difluoride (PVDF) membrane at 50 V for 90 min at a ice-cold temperature using Tris-glycine buffer (25 mM Tris, 192 mM glycine, 20% methanol), using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Inc.). Sera from 73 ACSs were obtained by venipuncture, stored at −80 °C, and flooded over the PVDF membrane at 1:200 dilution in 1% skim milk in Tris-Buffered Saline Tween 20.[21] Immunoreactivity was visualized with 3, 3', 5, 5′-tetramethylbenzidine and horseradish peroxidase conjugated to anti-rabbit IgG secondary antibody. A positive reaction was reported if staining was more intense than the negative control immunoblot reacted with the secondary antibody but no sera.

Two-dimensional electrophoresis and mass spectrographic analysis

Protein spots on the membrane were analyzed using the ImageJ (NIH) software. The molecular weights (MW) and isoelectric points (IP) of spots were estimated in triplicate using molecular weight markers (SDS7; Sigma) and 2-D SDS-PAGE standards (Bio-Rad). After the preparative gels were stained with Coomassie brilliant blue R-250 (Merck KGaA, Darmstadt, Germany), protein spots of interest were excised from the gels and digested with trypsin. The tryptic peptides were extracted and dried completely by centrifugal lyophilization. The peptides were analyzed by matrix-assisted laser desorption/ionization-time of flight-tandem mass spectrometry (MALDITOF-MS), performed using a Voyager-DE STR (Life Technologies, Tokyo, Japan). Mass spectra were used to search canine sequences in the NCBI nr database using the Mascot database search algorithms.

Statistical analysis

Statistical evaluations were performed with a commercially available software program (JMP10.0.2 statistical software; SAS Institute, Tokyo, Japan). The relationship between age and stage of cataract was evaluated by Spearman's rank correlation coefficient. Correlation between the incidence of βH-crystallins antibodies in sera and stages of cataract or uveitis were assessed by Pearson's χ^2-test. The Mann–Whitney *U*-test was performed to compare the incidence of uveitis in cataractous patients and normal controls. $P < 0.05$ was defined as statistically significant.

RESULTS

Evaluations of 73 ACS dogs found 12 with both lenses normal, three dogs with at least one lens with immature cataracts, 32 dogs contained mature cataracts, and 15 had hypermature cataracts (Fig. 1a). Lenses in 11 dogs could not be observed due to corneal opacity due to ocular surface abnormalities or glaucoma; these dogs were excluded from further analysis. To maintain statistical validity, the data from the very small group of patients with immature cataracts were also eliminated. The stage of cataract was found to be negatively correlated with age in our patient population ($P = 0.0054$, Fig. 1b).

In addition to cataract, uveitis was the major ocular disease in our study patients (Table 1a). Uveitis developed concomitantly with cataract, and this might be a reason why anti-inflammatory drugs were used in mature and hypermature cataract patients (Table 1b).

Purification of dog lens crystallins yielded the four known major peaks[22] for α-, βH-, βL, and γ-crystallins (data not shown), which contained several bands of crystallins (Fig. 2a). The polypeptides within the βH-crystallin group are comprised of seven separate gene products and their fragments,[20] and four arbitrary groupings of these protein bands (termed H1, H2, H3, and H4) based on their common range of MW's were used in a one-dimensional immunoblot assay for serum anti-βH-crystallin reactivity (Fig. 2b). No serum antibodies for any βH-crystallin were detected in approximately 30–40% of patients despite the existence of opacity (Fig. 3a, column '0'). This was highest frequency in the largest group with hypermature cataract (Fig. 3a, column '0', open bar). The group of patients with the highest frequency of mature cataracts (~40%), and the group with highest frequency of normal lenses (~50%) contained 2 and 3 kinds of antibodies for βH-crystallins, respectively. Serum antibody reactivity to groups of βH-crystallin polypeptides varied between groups of cataract patients (Fig. 3b).The incidence of serum antibodies against βH-crystallins in ACSs was not positively related to stages of cataract (Fig. 3b). Indeed, serum antibody reactivity against the β-crystallin H4 group was negatively correlated with stage of cataract ($P = 0.032$, Fig. 3b, H4*). Thus, the data do not confirm a positive relationship between serum βH-crystallin antibodies and stage of cataract.

Patients with cataract were also exhibited uveitis (Fig. 3c, 'Mature' and 'Hypermature'). The stage of uveitis was not different between patients with mature and hypermature cataracts.

Patients with normal lenses did not develop uveitis (Fig. 3c, 'Normal'). As with cataract, the stage of uveitis was negatively correlated with serum antibody reactivity against the β-crystallin H4 group ($P = 0.025$, Fig. 3d, H4*).

Protein staining on the two-dimensional proteome map of noncataractous Beagle βH lens crystallins detected 14 protein individual spots (Fig. 4a), varying in molecular weight from 22 to 27 kDa and ranging in IP from 5.4 to 6.4. After destaining these protein spots, immunoblot assays for serum βH-crystallin antibody activities in ACS dogs were performed. Antibody to the large amount of landmark Spot 6, identified as βB2-crystallin, was most frequently observed in 11 patients (normal plus cataractous). Interestingly, antibody reactions to spots 1, 5 and 13 were found only in the serum from patients with cataract (Fig. 4b*) and not in normal lenses (gray bars). Mass spectrographic analysis of these three spots indicated that they were from βA1-crystallin (Fig. 5).

DISCUSSION

A major finding of the present investigation was that serum antibodies to βH-crystallin in ACS dogs were not directly (positively) related to the stage of cataract

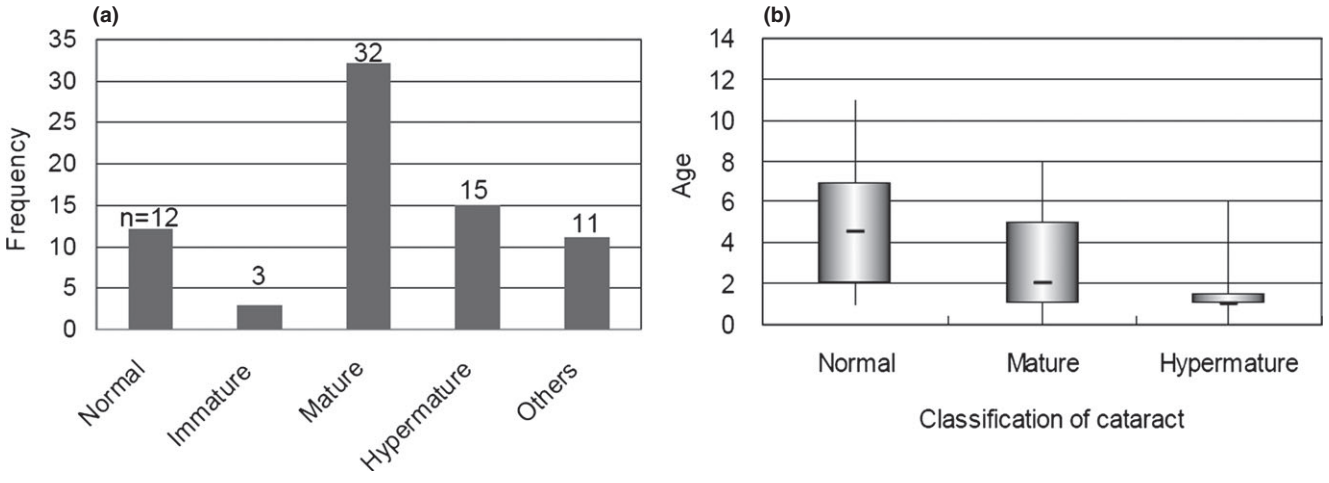

Figure 1. (a) Frequency of cataracts (worst eye) observed in ACS dogs in the present study. (b) Negative correlation ($P = 0.0045$) between stage of cataract and age depicted inbox and whisker plots showing median (–), minimum (top vertical bars), maximum (bottom vertical bars), upper and lower quartiles (tops and bottoms of boxes) for ages in each cataract group. The 'Others' group contained lenses not diagnosed due to corneal opacity by ocular surface abnormalities or glaucoma.

Table 1. Diagnosis (a) and medication (b) reported in the medical records for ACS dogs

(a)

		Disease											
Group	*n*	Male	Female	Uveitis	Uveal cyst	Glaucoma	Lens luxation	Retinal degerenation	Retinal degtachment	KCS	Keratitis	Eyelashes and eyelid diseases	Skin and ear diseases
Normal	12	6	6	0	0	0	0	0	0	1	0	2	3
Immature	3	1	2	0	0	0	0	1	0	0	0	0	0
Mature	32	19	13	23	3	0	0	0	0	0	1	2	5
Hypermature	15	8	7	9	0	0	0	0	1	0	0	1	3
Others	11	6	5	2	0	8	3	0	1	1	2	2	3

(b)

Group	*n*	Steroids	NSAIDs	Antioxidants	Antibiotics	Eyedrops for glaucoma	NSAIDs	Antioxidant supplement	CAIs
Normal	12	1	0	0	1	0	0	0	0
Immature	3	0	0	0	0	0	0	1	0
Mature	32	6	8	4	9	0	5	2	0
Hypermature	15	6	1	0	5	0	1	1	0
Others	11	0	1	0	4	7	1	1	4

NSAIDs, Non-steroid anti-inflammatory drugs; CAIs, Carbonic anhydrase inhibitors.

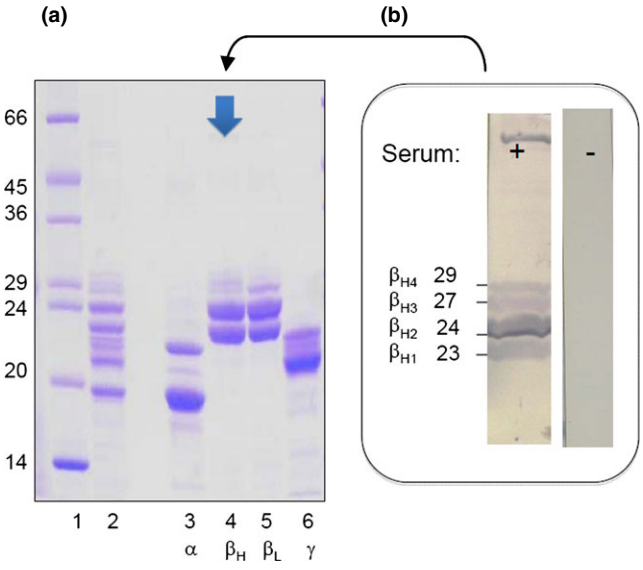

Figure 2. (a) SDS-PAGE of the crystallin peak fractions from Sepharose chromatography of Beagle dog water-soluble proteins used to isolate crystallins (Bold arrow indicates βH fraction used for further purification and testing). Lane 1: molecular weight marker, lane 2: total water-soluble proteins before purification, lane 3: α crystallin, lane 4: βH-crystallin, lane 5: βL crystallin, and lane 6: γ crystallin. (b) Immunoblots using sera from individual dogs blotted against the four numbered β_H bands at 23–29 kDa. (+) is a representative blot from a dog with serum antibodies reacting with all four bands; (−) is a negative control blot with no serum.

(Fig. 3b). We observed a significant decrease in anti-βH4-crystallin serum reactivity with stage of cataract. As cataract stage was negatively associated with aging (Fig. 1b), these ACS cataracts were likely due their known genetic propensity for cataract.[2] Due to heredity predilection in some breeds (Canine Inherited Disorders Database; URL http://ic.upei.ca/cidd/breed/cocker-spaniel-american [accessed on 06 September 2013]) show increased risk of certain disorders including cataract. Further, the data do not support a proposed mechanism whereby βH-crystallins leak from lenses due to membrane breakdown into the serum causing a generalized increased autoimmune reaction against lens proteins resulting in cataract. A similar negative correlation between serum antibodies against general lens crystallins and stage of cataract in an unknown species of dog was also reported,[12] and the data supporting a similar autoimmune mechanism for human cataract are conflicted.[23–25]

Another major new finding was presentation of a proteome map for normal, canine βH-crystallins (Fig. 4a). Features included the predominance of reactivity for the abundant lens crystallin βB2. We noted 14 different polypeptides with differing masses and PI's. As only seven separate gene products exist for the βH-crystallin family, some of the spots were due to degradation and/or protein modifications of the βH-crystallin polypeptides. For example, spots 1, 5 and 13 were only found in sera from dogs with cataractous lenses and showed three masses of 26.9, 22.3, and 23.7 kDa, respectively. These spots were determined to be βA1-crystallin (Fig. 5), which has a native molecular weight of 22.3 kDa.

The β-crystallin family consists of four acidic (βA1-, βA2-, βA3-, βA4-crystallin) and three basic (βB1-, βB2-, and βB3-crystallin) protein members that form homodimers and then aggregate into complex hetero-oligomers. Heterodimers of β-crystallins increase during aging and cataracts, contributing consequently to the accumulation

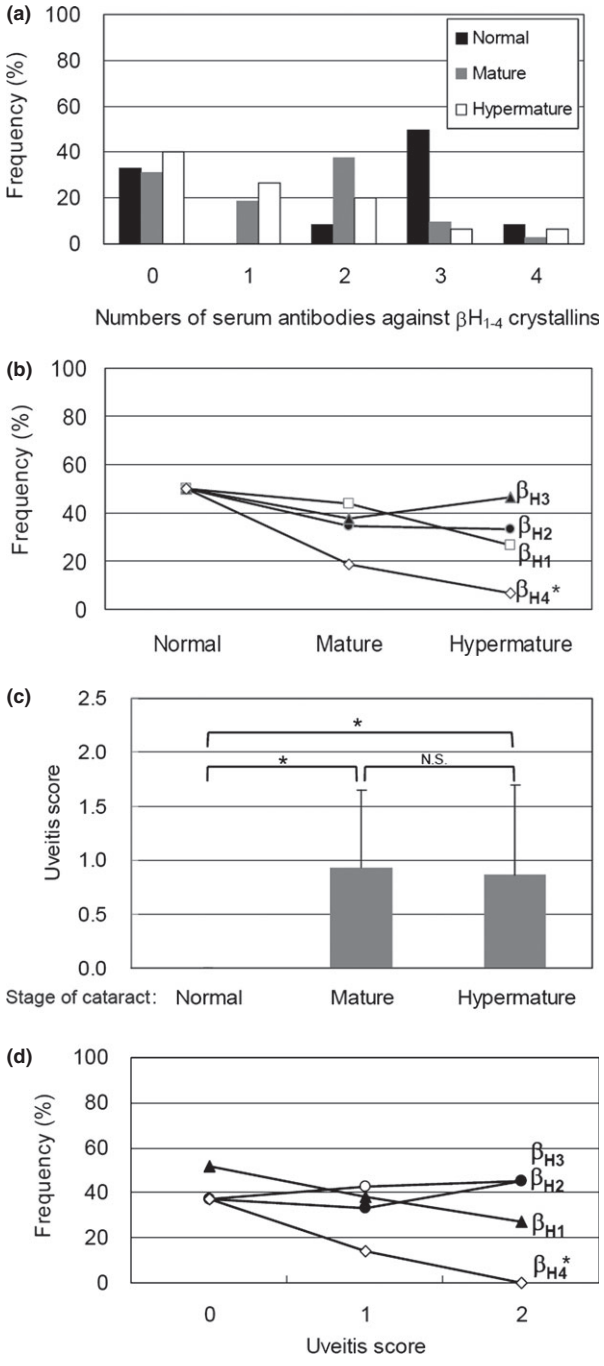

Figure 3. (a) The% frequency of the total number of positive antibodies against βH subunits existing in the serum of ACS dogs with and without cataracts. (b) Lack of relationship ($P > 0.05$) between the frequency of positive serum antibody reactions against $\beta_{H1\text{-}3}$ bands and the stage of cataract. Note that serum antibody against β_{H4} was negatively associated with the stage of cataract (*$P < 0.05$). (c) Positive relationship between the stage of cataract and the uveitis score. Data are means ± SD (n = 12–32). *$P < 0.05$ relative to normal (Mann–Whitney U-test); n.s. indicates not statistically significant. (d) Lack of relationship ($P > 0.05$) between frequency of positive serum antibody reactions against $\beta_{H1\text{-}3}$ bands and the stage of uveitis. Note that serum antibody againstβ_{H4} was negatively associated with the more advanced stage of uveitis (*$P < 0.05$).

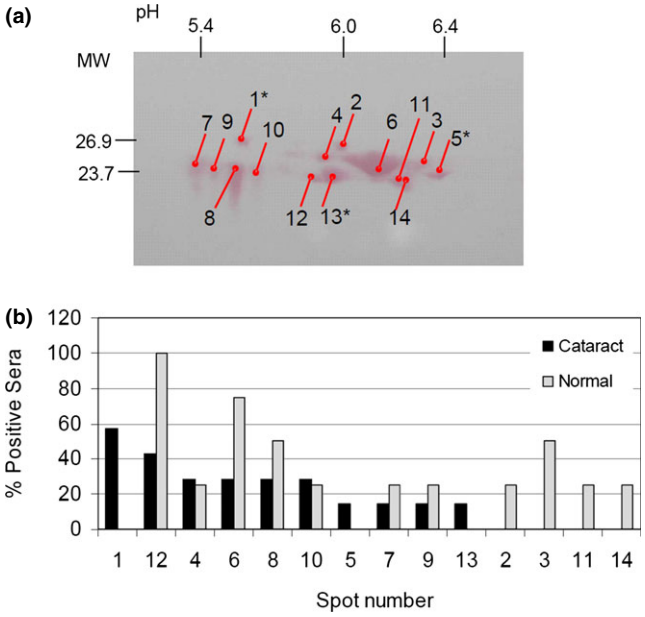

Figure 4. (a) Two-dimensional proteome map of βH-crystallins from Beagle dogs with normal lenses. Numbers were arbitrarily assigned to spots to facilitate discussion in the Figures below. (*) = Spot analyzed by mass spectrometry. (b) Frequency of ACS patients with antisera showing positive reactions against βH-crystallin spots 1–14 shown in Fig. 4a. Note that reactivity to spots 1, 5, and 13 was only found in sera from patients with cataract (black bars) and not in normal lenses (grey bars).

of insoluble β-crystallins.[11,26,27] The production of antibodies against β-crystallins may be related to the instability of dimer formation of β-crystallins.

Increased calcium is present in many types of cataract.[8,28,29] Three types of calcium-activated proteases (calpain 1, calpain 2, and Lp82) are present in dog lenses (Y. Tamada, T.R. Shearer, M. Azuma, unpublished data), and we speculate that calpain may have caused increased degradation in ACS cataracts. Thus, while levels of antibodies to groups of βH-crystallins was not related to cataract, release of specific polypeptides, such as βA1-crystallin or its derivatives, may have been increased, and thereby increased serum antibodies to them in ACS cataract. Although more samples are needed, the data suggested that increased reactivity by βA1-crystallin antibodies is associated with cataract formation. If future studies detect anti-βA1-crystallin antibodies at the earlier stages of cataract formation, the antibody may be useful as a prognostic marker for earlier cataract treatment and prevention. This is especially pertinent as βA1-crystallin is one of the most abundant crystallins to be insolubilized in cataractous lenses.[14,30]

ACKNOWLEDGMENTS

The authors thank Yumiko Nishina and Hiroki Takahashi, graduate students in Azabu University, for help in a part of data analysis. We also thank Hideyuki Sakaki, PhD,

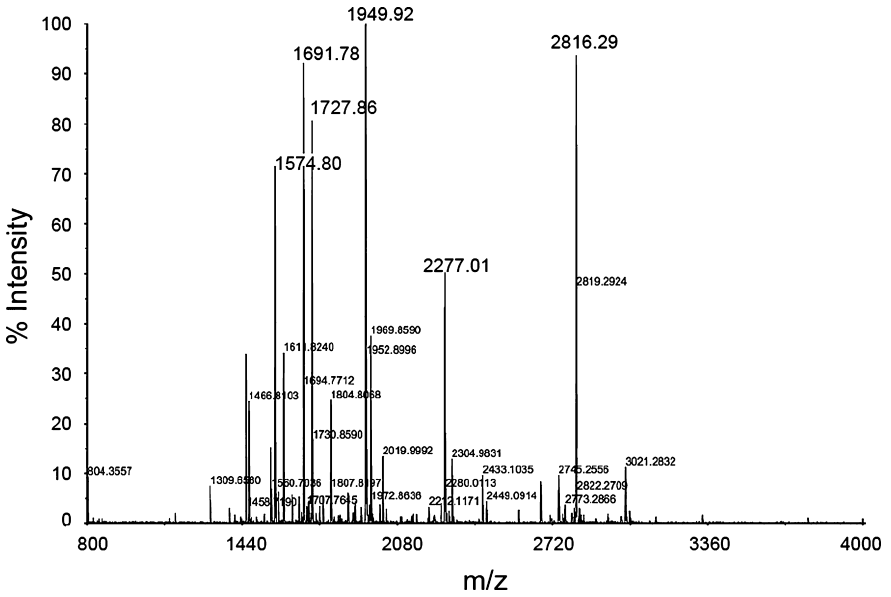

Figure 5. MALD-TOF MS spectra of the tryptic fragments from spot 1. All spots 1, 5, and 13 were identified as originating from βA1 crystallin.

Senju Pharmaceutical Co Ltd, Kobe, Japan for his statistical consultation.

AUTHORS' DISCLOSURE STATEMENT

Dr. Shearer is a paid consultant for Senju Pharmaceutical Co. Ltd., a company that may have a commercial interest in the results of this research and technology. Dr. Azuma is an employee of Senju Pharmaceutical Co., Ltd. These potential conflicts of interest were reviewed, and management plans approved by the OHSU Conflict of Interest in Research Committee were implemented.

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