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Accumulation of the hydroxyl free radical markers meta-, ortho-tyrosine and DOPA in cataractous lenses is accompanied by a lower protein and phenylalanine content of the water-soluble phase

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Abstract

Post-translational modifications of lens proteins play a crucial role in the formation of cataract during ageing. The aim of our study was to analyze protein composition of the cataractous lenses by electrophoretic and high-performance liquid chromatographic (HPLC) methods.

Samples were obtained after extracapsular cataract surgery performed by phacoemulsification technique from cataract patients with type 2 diabetes mellitus (DM CAT, $n = 22$) and cataract patients without diabetes (non-DM CAT, $n = 20$), while non-diabetic non-cataractous lenses obtained from cadaver eyes served as controls (CONTR, $n = 17$). Lens fragments were derived from the surgical medium by centrifugation. Samples were homogenized in a buffered medium containing protease inhibitor. Soluble and insoluble protein fractions were separated by centrifugation. The electrophoretic studies were performed according to Laemmli on equal amounts of proteins and were followed by silver intensification. Oxidized amino acid and Phe content of the samples were also analyzed by HPLC following acid hydrolysis of proteins.

Our results showed that soluble proteins represented a significantly lower portion of the total protein content in cataractous lenses in comparison with the control group (CONTR, 71.25%; non-DM CAT, 32.00%; DM CAT, 33.15%; $p < 0.05$ vs CONTR for both). Among the proteins, the crystallin-like proteins with low-molecular weight can be found both in the soluble and insoluble fractions, and high-molecular weight aggregates were found mainly in the total homogenates. In our HPLC analysis, oxidatively modified derivatives of phenylalanine were detected in cataractous samples. We found higher levels of m-Tyr, o-Tyr and DOPA in the total homogenates of cataractous samples compared to the supernatants. In all three groups, the median Phe/protein ratio of the total homogenates was also higher than that of the supernatants (total homogenates vs supernatants, in the CONTR group 1102 vs 633 μmol/g, in the DM CAT group 1187 vs 382 μmol/g and in the non-DM CAT group 967 vs 252 μmol/g; $p < 0.05$ for all).

In our study we found that oxidized amino acids accumulate in cataractous lenses, regardless of the origin of the cataract. The accumulation of the oxidized amino acids probably results from oxidation of Phe residues of the non-water soluble lens proteins. We found the presence of high-molecular weight protein aggregates in cataractous total homogenates, and a decrease of protein concentration in the water-soluble phase of cataractous lenses. The oxidation of lens proteins and the oxidative modification of Phe residues in key positions may lead to an altered interaction between protein and water molecules and thus contribute to lens opacification.

Keywords: *Cataract, phenylalanine, meta-tyrosine, ortho-tyrosine, 3,4-dihydroxy-phenylalanine, protein solubility*

Abbreviations: *ATP, adenosine triphosphate; AUC, area-under-the-curve; CBB, Coomassie brillant blue; CONTR, group of control subjects; DM, diabetes mellitus; DM CAT, group of cataract patients with type 2 diabetes mellitus; DOPA, 3-, 4-dihydroxy-phenylalanine; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-tetraacetic*

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acid; ELFO, electrophoresis; Hb A_{1c}, hemoglobin A_{1c}; HPLC, high performance liquid chromatography; m-Tyr, meta-tyrosine; non-DM CAT, group of cataract patients without diabetes mellitus; o-Tyr, ortho-tyrosine; p-Tyr, para-tyrosine; PAGE, polyacrylamide gel-electrophoresis; Phe, phenylalanine; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecylsulphate; Tyr, tyrosine; UV, ultraviolet

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Introduction

The formation of cataract due to an increased opacity of the lens is the most common cause of blindness. The lens is a unique organ in many regards, since it is avascular, it has a high cellular protein concentration, despite the high protein concentration still transparent for visible light, and it has an extremely low protein turnover [1]. Since the lens is avascular, i.e. possesses no direct blood supply, and is surrounded by the lens capsule, it represents a unique, relatively closed system. As a consequence of the low protein turnover, the physico-chemical effects from the inner and outer environment on the lens cumulate and lead to the development of opacification with age [2]. The cataractogenic effects of ultraviolet (UV) light, ionizing radiation, pharmacological agents, smoking and diabetes mellitus (DM) are well-known [3–5], but the exact pathogenesis of cataract formation is a complex process with many still unknown components. It is known that, as a consequence of the physico-chemical impacts, the dynamic interactions between water, inorganic ions and proteins (representing 99% of the dry weight) disintegrate, and the balance between the tissue and the environment suffers disturbances [6]. On the level of proteins, irreversible damages evolve, which affect the cytoskeletal system of the lens, as well as the crystallin-type proteins. Glycation, carbonylation, formation of cross-links, oxidation of thiol-groups and formation of aggregates finally lead to insolubilisation of proteins [7–9], and, therefore, opacity develops.

Hydroxyl free radicals formed in oxidative stress processes give rise to the conversion of phenylalanine (Phe) to three different tyrosine (Tyr) isomers, i.e. para-, meta-, and ortho-tyrosine (p-Tyr, m-Tyr, o-Tyr). p-Tyr is the physiologic isoform that is produced by an enzymatic reaction (phenylalanine hydroxylase), as well. In contrast to that, the other two isoforms (m-Tyr and o-Tyr) are present in very low concentrations under physiologic circumstances, therefore, their detection is regarded as a valuable method to study the hydroxyl free radical-related oxidative damage [10]. In a further enzymatic or hydroxyl free radical-driven reaction 3,4-dihydroxi-phenylalanine (DOPA) is formed from p-Tyr. In the cataractous lens, protein-bound DOPA is regarded as a marker of hydroxyl radical-mediated damage, as well [11]. However, DOPA may be degraded to DOPA-quinones due to further free radical-caused damage.

High glucose concentration evokes oxidative stress among others due to the damaged intracellular metabolism, the poliol-pathway and non-enzymatic glycation reactions. In non-diabetic subjects, free radicals formed during ageing processes may also contribute to age-related cataract formation [12].

In our paper, we present novel results on the comparison of proteins of non-cataractous, ageing and diabetic cataractous lenses. In our article we show the electrophoretic studies of the soluble and insoluble protein fractions in one hand, and we present post-translational modifications of amino acid residues on the other hand. Changes due to oxidative stress were investigated by high performance liquid chromatographic (HPLC) technique by measuring the levels of Phe, and amino acids formed by the oxidative modification of Phe.

Patients and methods

In a cross-sectional study we examined human lenses that were removed during extracapsular cataract surgery, upon informed consent. All cataractous lenses were removed under the same circumstances by the same surgeon. Cadaver eyes enucleated for corneal transplantation served as controls. The study was approved by the Ethical Board of the Medical Faculty of the University of Pécs.

Three groups were formed, a group of non-diabetic non-cataractous controls (CONTR), patients with cataract and type 2 diabetes mellitus (DM CAT), and a group of patients with cataract without diabetes (non-DM CAT). Type 2 DM has been diagnosed upon diagnostic criteria of the American Diabetes Association (ADA). There was no difference between the age of the CONTR and the DM CAT groups; however, patients in the non-DM CAT group were significantly older than both the CONTR and the DM CAT groups. This finding is not surprising, as progredient phase of senile cataract (i.e. in the non-DM CAT group) develops at older age than diabetic cataract of the same phase (i.e. in the DM CAT group). Main clinical characteristics of the patients are shown in Table I.

Surgery was carried out under topical anaesthesia through a 3.2 mm long clear corneal incision after dilation of the pupil. Capsulorhexis was performed under the infusion of a viscoelastic material and was followed by hydrodissection and rotation of lens nucleus. The lens nucleus was removed by a "divide

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Table I. Clinical characteristics of the patients.

	CONTR	DM CAT	Non-DM CAT
Number of cases	$n = 17$	$n = 22$	$n = 20$
Age (years)	62 (48–73)	67 (64–78)	75*,** (71–82)
HbA_{1c} (%)		6.74 (6.35–7.64)	
Fructosamine (μmol/l)		272 (227–334)	
Fasting plasma glucose (mmol/l)		8.10 (7.03–9.23)	

*$p < 0.05$ vs CONTR; **$p < 0.05$ vs DM CAT; CONTR, group of control subjects; DM CAT, group of cataract patients with type 2 DM; non-DM, group of cataract patients without DM; Hb A_{1c}, hemoglobin A_{1c}. Data are given as median (inter-quartile range).

and conquer" phacoemulsification technique, the cortex by irrigation-aspiration. The posterior capsule was polished and vacuum-cleaned when needed, and a foldable artificial lens was implanted into the capsular bag under viscoelastic material, after wound enlargement. Viscoelastic material was then washed out thoroughly and operation was finished with subconjunctival gentamycine and dexamethasone injection. Cadaver lenses were extracted from the eye, decapsulated, and were subsequently transported in a buffered medium. Further process was carried out without delay.

Protein solubility and distribution

The specimen from the surgical medium was extracted immediately after surgery by centrifugation for 30 min at 13,000 rpm. The obtained pellet was resuspended in a Tris-buffered solution (pH 6.8) containing ethylenediamine-tetraacetic acid (EDTA, 1 mmol/l), ethylene glycol-bis(2-aminoethylether)-tetraacetic acid (EGTA, 4 mmol/l) and phenylmethanesulfonyl fluoride (PMSF, 0.2 mmol/l). After this, both the cataractous and the control samples were treated and homogenized the same way. Assessment of protein concentration of the total homogenate was carried out according to Bradford from aliquot amounts of samples following repeated homogenization. The water-soluble (i.e. physiologic) and the water-insoluble (i.e. damaged) components of the lenses were separated from each other by centrifugation (13,000 rpm, 30 min). Soluble protein fractions were gained as the supernatant of equal amounts of the homogenates of each sample. The protein concentration of the supernatants was assessed, as well. The ratio of soluble proteins was calculated as percent of the total protein content. Until further process, the homogenates and supernatants were stored at − 70°C. The same samples were used for the investigation of protein distribution, for protein electrophoresis and HPLC analysis.

Gel-electrophoretic studies

Separation of proteins was performed by sodium-dodecylsuphate polyacrylamide gel-electrophoresis (SDS-PAGE) according to Laemmli [13]. Comparative PAGE was performed both from proteins of the total homogenate and the supernatant proteins. Laemmli buffer was added to proteins. A measured quantity of 3–3 ig protein per line was analyzed in a 12.5% gel in a Mini Protean 3 Cell. Low molecular weight proteins were used as markers. Detection of protein fractions was performed by silver post-intensification according to Willoughby [14] following the traditional Coomassie brilliant blue (CBB) R-250 staining. After documentation of the electrophoretograms, the stained gels were assessed by direct densitometry.

High performance liquid chromatographic analysis

The measurement of amino acid composition of lens proteins was carried out in an HPLC analysis by applying the method described by Ishimitsu et al. [15] for the lens samples. In well-closing, O-ring protected polypropylene tubes 200 μl of the samples was measured, subsequently 4 μl of 400 mM desferrioxamine (final concentration: 3.6 mM) and 40 μl of 500 mM butylated hydroxytoluene (final concentration: 45 mM) were added to avoid possible free radical formation during hydrolysis. Then 200 μl of 12 N hydrochloric acid was added, and we performed an overnight acid hydrolysis of the proteins at 120°C.

The hydrolyzate was then filtered through a 0.2 μm filter (Millipore Inc.) and 20 μl of the filtrate was injected onto the HPLC column of a Shimadzu Class LC-10 AD_{VP} HPLC device (Shimadzu Manufacturing Inc., USA) using a Rheodyne manual injector. Quantitative analysis of the amino acids was carried out using a Licrospher-C_{18} silica column (Merck) upon their autofluorescence. Tyr isomforms and DOPA were measured at 275 nm excitation and 305 nm emission, while Phe at 258 nm excitation and 288 nm emission using a Shimadzu RF-10A_{XL} fluorescent detector. Therefore, no pre-column or post-column staining or derivatization was required. An isochratic HPLC run was performed, the eluent contained 1% acetic acid, 1% sodium-acetate and 98% distilled water.

The area-under-the-curve (AUC) was determined for the amino acids, and exact concentrations were

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calculated using external standard calibration. In some cases the elution time of the substances was also verified by standard peak-addition method. The amino acid concentrations were corrected for protein content or Phe concentrations.

Statistical analysis

Statistical analysis was performed using the SPSS 10.0 software (SPSS Inc., IE, USA). As most data were of non-normal distribution, we used median and interquartile range to characterize distribution of the data and Kruskal-Wallis test and Mann-Whitney U-tests were used for between-group comparisons.

Results

Protein distribution

We found that the protein distribution between the soluble and non-soluble phases is different, i.e. the ratio of soluble proteins is lower in cataractous lenses compared to the CONTR group. There was no significant difference between the DM CAT and non-DM CAT groups, both had equally low soluble protein content (Figure 1).

Results of the electrophoretic analyses

Proteins were separated depending on their molecular weight in each sample with comparative eletrophoretic (ELFO) studies. Equal protein amounts were compared. This way, similarities and differences between protein composition were well judgeable. Analysis was performed both from total and soluble protein fractions. Among lens proteins, the presence of proteins in the range 20–30 kDa was characteristic for each sample. According to densitometric measurements, this group represented approximately 30% of the electrophoretized proteins of CONTR samples.

Figure 2 shows electrophoretograms of representative samples. The differences among the protein pattern of the 20–30 kDa group are striking. In each sample, there are several visible bands in this area, but the pattern and the intensity of the bands is different. Among the visible bands, the two bands nearest to the 20 kDa marker (marked with white arrowheads in lane 6), are present in each sample, and they show a high intensity in each sample. However, in the total homogenates of cataractous lenses (lanes 3 and 5), close to the 30 kDa marker, two additional bands—marked with white arrows in lanes 3 and 5—also become quantitatively dominant. These bands are either not present in the supernatants and the control sample, or they show less intensity.

Moreover, it is worth mentioning that in all total homogenates we observed staining, which indicates the presence of high molecular weight aggregates, at the dividing line between stacking and separating gel and in the stacking gel itself (see the >94 kDa area in Figure 2). Proteins in the stacking gel are mainly stained in the total homogenate of the cataractous samples, and less in the control sample (see lanes 3 and 5 vs lane 1). The presence of high molecular weight aggregates is less characteristic for the supernatants (compare total homogenates vs lens supernatants; i.e. lane 1 vs 2, lane 3 vs 4, lane 5 vs 6; Figure 2).

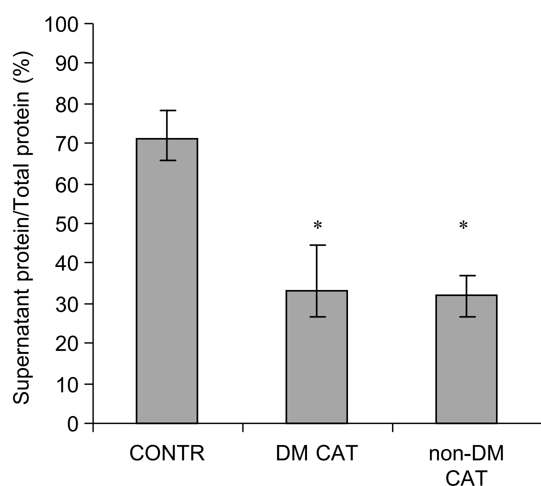

Figure 1. Protein distribution between the supernatant fractions and the total homogenates calculated by the ratio of protein contents of the supernatant and total homogenates. CONTR, group of control subjects; DM CAT, group of cataract patients with type 2 DM; non-DM CAT, group of cataract patients without DM. *$p < 0.05$ versus CONTR. Values are represented as median (interquartile range).

Results of the HPLC analyses

The concentration of total lens homogenates was set to 1 mg/ml using the homogenizing buffer, while in the lens supernatants of the three groups there were higher protein concentrations (medians: 1.45–2.50 mg/ml). Amino acid concentrations of the solutes were thus corrected for sample protein content.

Using the HPLC method with fluorescent detection, the amount of DOPA, p-Tyr, m-Tyr, o-Tyr as well as the parent amino acid Phe can theoretically be measured in the same run of the samples. In our experimental system, the amount of p-Tyr was not measured, as its concentration exceeds that of both DOPA and m-Tyr by approximately three orders of magnitude. Two representative chromatograms are shown in Figure 3.

The amount of m-Tyr was below detection limit in a majority of supernatant samples. By comparing the groups in this regard, we found that detectability of m-Tyr was higher in the supernatants of the non-DM CAT (18/22 cases, detectable/all cases) group than in the CONTR (7/17 cases) and the DM CAT

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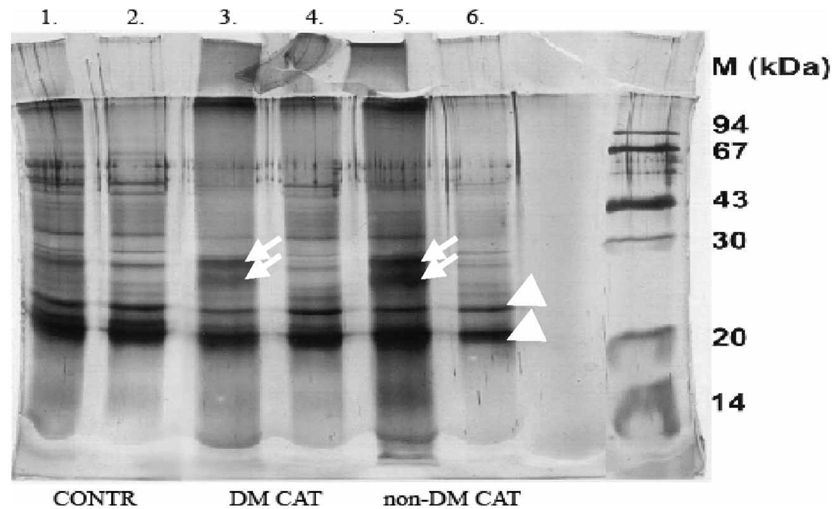

Figure 2. Sodium dodecylsulphate-polyacrylamide electrophoretogram (SDS-PAGE) of proteins of representative lens samples. Bands 1 and 2 , CONTR samples; bands 3 and 4, DM CAT samples; bands 5 and 6, non-DM CAT samples; bands 1, 3, 5, total homogenates; bands 2, 4, 6, supernatants. CONTR, group of control subjects; DM CAT, group of cataract patients with type 2 DM; non-DM CAT, group of cataract patients without DM. Arrows and arrowheads: see explanation in the text.

(10/20 cases) groups (K-W test, $p < 0.05$; M-W U test, non-DM CAT vs CONTR, $p < 0.05$; non-DM CAT vs DM CAT, $p < 0.05$; χ^2-test $p < 0.05$). In the total homogenates we found that detectability of m-Tyr was higher in the DM CAT (20/20 cases) and non-DM CAT groups (22/22 cases) than in the CONTR (11/17 cases) group (K-W test, $p < 0.05$; M-W U test, DM CAT vs CONTR, $p < 0.05$; non-DM CAT vs CONTR, $p < 0.05$). In case of the total homogenates, χ^2 test could not be performed for m-Tyr detectability and the patient groups because of mathematical reasons (expected number of cases per cell was too low). In the case of m-Tyr, descriptive and comparative statistics could only be calculated for those samples, where m-Tyr was detectable (Table II).

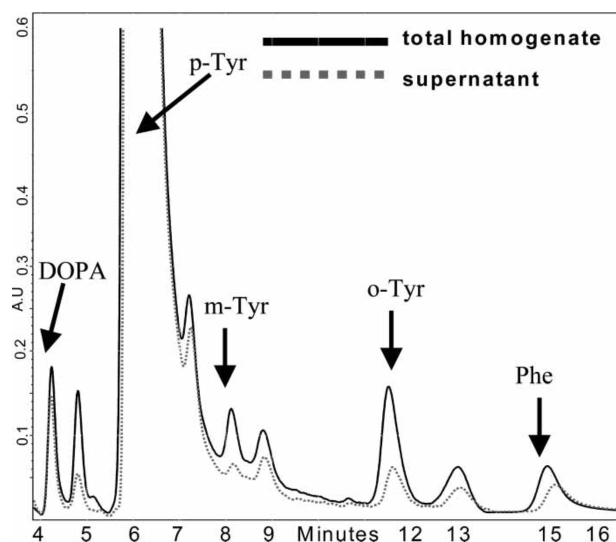

Figure 3. HPLC registrate of the total homogenate (continuous line, 1 mg/ml protein) and the supernatant lens fraction (dotted line, 2.5 mg/ml protein) of a cataract patient with type 2 DM. DOPA, 3,4-dihydroxy-phenylalanine; p-Tyr, para-tyrosine; m-Tyr, meta-tyrosine; o-Tyr, ortho-tyrosine; Phe, phenylalanine.

Inter-group comparisons (amino acid/protein)

Concentration of the amino acids was expressed as their ratio to sample protein content, i.e. DOPA/protein, m-Tyr/protein, o-Tyr/protein and Phe/protein ratios were calculated.

In the lens supernatants, DOPA and o-Tyr could be well detected, however, in a majority of the supernatant samples, m-Tyr was below the detection limit. When comparing the three groups, we found that there was no difference between DOPA/protein, m-Tyr/protein and o-Tyr/protein ratios of the supernatants of the three groups. The Phe/protein ratio of the DM CAT samples was not significantly lower than that of the CONTR samples. However, in the supernatants of the non-DM CAT group we found a significantly lower Phe/protein ratio than in the CONTR group.

In the lens total homogenates, DOPA and o-Tyr could be measured, as well. In the majority of total homogenates of the CONTR samples, m-Tyr was below the detection limit, while it could be detected well in the cataractous samples. There was no difference between DOPA/protein ratio of the total homogenates of the three groups. However, m-Tyr/protein and o-Tyr/protein of both DM CAT and non-DM CAT groups was higher than that of CONTR samples. In the total homogenates, there was no difference in the Phe content among the groups (Table II).

The difference between Phe content of proteins of the supernatant and the total homogenate was calculated for all groups by dividing Phe/protein of

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Table II. Amino acid contents of the supernatant samples and the total homogenates corrected for sample protein content.

		CONTR	DM CAT	Non-DM CAT
Supernatant samples	DOPA/protein (nmol/g)	413.56 (79.01–1505.84)	305.53 (196.99–409.63)	188.61 (114.00–245.42)
	m-Tyr/protein† (nmol/g)	1.06 (0.77–3.01)	3.20 (1.22–4.74)	2.46 (1.54–4.47)
	o-Tyr/protein (nmol/g)	30.96 (3.39–37.08)	22.43 (7.82–87.99)	18.66 (11.17–121.84)
	Phe/protein (μmol/g)	633 (299–819)	382 (332–512)	252*,** (197–306)
Total homogenates	DOPA/protein (nmol/g)	1100.35 (289.34–1767.30)	904.12*** (738.49–1287.17)	1346.23*** (428.44–738.49)
	m-Tyr/protein† (nmol/g)	3.41*** (3.02–4.61)	15.34*, *** (2.39–29.65)	20.28*, *** (7.18–57.29)
	o-Tyr/protein (nmol/g)	38.61 (12.47–51.64)	217.58*, *** (22.76–919.10)	211.78*, *** (51.76–1128.03)
	Phe/protein (μmol/g)	1102*** (601–1246)	1187*** (956–1622)	967*** (626-–336)

*$p < 0.05$ vs CONTR; **$p < 0.05$ vs DM CAT; ***$p < 0.05$ vs supernatant. CONTR, group of control subjects; DM CAT, group of cataract patients with type 2 DM; non-DM, group of cataract patients without DM; DOPA, 3,4-dihydroxy-phenylalanine; m-Tyr, meta-tyrosine; o-Tyr, ortho-tyrosine; Phe, phenylalanine;. Values are represented as median (inter-quartile range).
† Amount of m-Tyr in some samples was below detection limit, statistics were only calculated for those samples, in which m-Tyr was in the detection range.

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the supernatant with Phe/protein of the total homogenate: the median ratio of the CONTR group was 67%, and there was a lower ratio in the DM CAT and non-DM CAT groups (26 and 30%, respectively, $p < 0.05$ vs CONTR, for both).

Comparison of the total homogenates with the supernatants

Besides the inter-group comparison, also intra-group comparison of supernatants and total homogenates of the same samples was carried out. In the CONTR, DM CAT and non-DM CAT groups, composition of total homogenates was compared to composition of the supernatants.

We found that the total homogenates and supernatants of CONTR samples did not differ in their DOPA/protein ratio, however, in the DM CAT and non-DM CAT groups there was a higher DOPA/protein ratio in total homogenates compared to supernatants.

m-Tyr could be measured well in total homogenates of DM CAT and non-DM CAT lenses. The m-Tyr/protein ratio of the total homogenates was higher in all three groups than that of the supernatants.

Compared to the supernatants, the o-Tyr/protein ratio was higher in the total homogenates of the DM CAT and non-DM CAT groups, but not the CONTR.

In all three groups, the Phe/protein ratio of the total homogenates was higher than that of the supernatants (Table II).

Discussion

The dynamic, close interaction taking place between lens components plays a crucial role in the maintenance of lens transparency. During ageing, the water, ion and protein content of the lens changes even under physiologic conditions. As long as the protecting mechanisms of the lens are able to counterbalance the physico-chemical influences of the environment, the lens remains transparent. Development of cataractous lens always indicates that harmful influences exhaust defending mechanisms of the organ. In this process, low molecular weight chaperone proteins, adenosine triphosphate and proteins of the cytoskeletal system play a distinguished role [16]. The high dry material content of the lens is in a close interaction with water. According to magnetic resonance imaging studies, the majority of the water content of the lens is so-called bound water, and its ratio varies inside the lens as well. Changes in the total water content and significant decrease of bound water has been well proven [17]. All the changes taking place in the soluble and insoluble fractions of the lens proteins are simultaneously reflected in a shift of the ratio of the bound water fraction. In such cases, the denatured proteins loose their close relation with water, protein–protein associations alter and form molecular aggregates [18].

In our study, we investigated the protein distribution, ELFO picture and oxidized amino acid content of non-cataractous and cataractous lenses. This way we could compare the lens composition of the non-cataractous controls, patients with diabetic cataract and senile cataract. By comparing the water-soluble supernatant and the total lens homogenate, we intended to find connection between water-solubility and lens composition.

According to our findings, protein content of the soluble fraction was lower than that of the total homogenates, and the soluble protein content of

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cataractous lenses showed a significant decrease when compared to controls. Between the different types of cataracts (DM CAT and non-DM CAT), we have not found any significant difference.

Analyzing equal amounts of proteins, control samples were the most heterogeneous. The range between 20 and 30 kDa deserves special interest. Protein fractions in the 20 kDa area are presumably alpha-crystallin subunits. Comparing control fractions with the cataractous samples the most striking differences are observed in the 20–30 kDa range. In the range of the medium weight, soluble proteins of control samples show a rich pattern, while the same fractions of cataractous lenses show a poor pattern. It is worth mentioning that in the high molecular weight region, we could identify the presence of high molecular weight aggregates in the total homogenates. This fraction is less characteristic for the lens supernatants. Our studies on proteins confirm literary data that refer to crystallin insolubilisation and cytoskeletal reconstruction during cataractogenesis [19,20].

The HPLC method used in our study is capable of detecting the autofluorescent amino acids in the homogenates of cataractous lenses. The presence of the Phe-derived free radical marker amino acids DOPA and o-Tyr could be verified and their concentrations could be measured and compared in the supernatants and the total lens homogenates. The amount of m-Tyr was below detection limit in a high proportion of the CONTR total samples, and could not be determined in a larger proportion of the supernatant samples. It is well-known [21], and in other experiments we have also found (data not published), that oxidized Phe derivatives are formed in nearly equimolar concentrations *in vitro*, but their formation might be different *in vivo* [21]. Therefore, there can be concentration differences between m-Tyr and o-Tyr of up to ten times [21].

It is widely accepted and in fact recommended to correct the amount of the oxidized amino acid to the amount of the parent amino acid (in our case to Phe) [21]. However, we found that there is a significant difference in Phe content of the proteins in the lens supernatant and in the proteins of the total homogenates. We also found that Phe content of the supernatants changes from controls to cataract samples. If we calculate the ratio of oxidized amino acid/Phe, it may be influenced to a larger extent by the difference in the amount of Phe than by the amount of the oxidized amino acids. Therefore, we used the amino acid/protein ratio for comparisons. In our study, the o-Tyr/Phe ratio for the total homogenate of control lenses vs senile cataractous lenses (0.034 vs 0.69 nmol o-Tyr/μmol Phe) roughly corresponds the range described in the literature, i.e. 0.18 vs 0.65 nmol o-Tyr/μmol Phe [11] and 0.5 vs 1.8 nmol o-Tyr/μmol Phe [21] (data not shown in table).

In the HPLC studies, we have observed the accumulation of the oxidized amino acids in the proteins of total homogenates of the cataractous samples, which process is not accompanied by an accumulation of the same hydroxyl radical markers in the supernatant of the cataract samples. These two findings provide an indirect proof that the accumulation of the oxidative stress markers occurs in the non-water soluble phase of the cataractous lens proteins.

The Phe/protein ratio is influenced by the consumption of Phe by only 0.1–0.01%, while the Phe content of the DM CAT and non-DM CAT samples is 40 and 60% lower than that of the control samples (Table II, supernatant data). The different Phe content of the proteins of the supernatants and the total homogenates, and the lower Phe content in the cataractous supernatants—versus that of the controls—is probably not a consequence of the consumption of Phe by the hydroxyl radical-derived processes, as there is a difference of approximately three-four orders of magnitude between protein Phe content and protein m- and o-Tyr content (Phe vs m-Tyr and o-Tyr, approximately 1,000 μmol/g vs 20 and 200 nmol/g, Table II). Moreover, we measured a lower Phe/protein ratio in all supernatants without the accumulation of the oxidized amino acids. There was a further decrease of Phe content in the non-DM CAT group vs controls in the supernatants. This is not accompanied by an increase in the amount of oxidized amino acids (Table II). These facts further support that the difference in Phe content is not a consequence of the oxidation of Phe. Thus, the difference in the Phe content might rather suggest that different types of proteins can be found in the water-soluble and in the water-insoluble lens components. It is known that amino acid composition of lens proteins is heterogenous among different parts of the lens, and in fact, the Phe and Tyr content may be used to distinguish between different crystallin proteins (α, β, γ) and to monitor maturation of lens proteins [22]. Our data obtained by calculating the supernatant Phe/total homogenate Phe ratio, also indicate that proteins in the supernatants and the total homogenates are different, as reported by the literature [23].

The major components of lens proteins are crystallins that play an important role in keeping the solution of high-protein concentration transparent. Investigation of m- and o-Tyr content of the lens proteins deserves special interest because of two reasons: *i.* The oxidized derivatives of Phe are selective markers of the presence of hydroxyl free radical. The accumulation of m- and o-Tyr is shown to run parallel to the accumulation of other oxidative stress-related processes, such as disulphide-bond formation, glycoxydation and the presence of cross-links [24] which are shown to play a role in the insolubilisation of lens proteins. *ii.* m- and o-Tyr are also noteworthy because they are markers of the oxidative modification of an

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important amino acid, Phe. Phe is shown to play a pivotal role in the solubility and chaperone funtion of crystallins [25,26]. On the one hand, there is a highly conserved SRLFDQFFG sequence in the N-terminal domain of αA- as well as αB-crystallin and other heat shock proteins. The Phe (F) residues together with the Leu (L) residue form a hydrophobic core that have been shown to play a crucial role in inter-subunit interactions and oligomere formation, stabilization of secondary, terciary and quaternary protein structure and chaperone function of α-crystallins [25]. It has also been proven that there is another Phe in a key position, namely the ^{71}Phe of αA-crystallin. Site-directed, selective mutagenesis of this single amino acid has been shown to completely abolish chaperone function of αA-crystallin [26]. Hydroxyl radical-related modification of the side chains of the Phe amino acids in key positions for the chaperone function of crystallins might also influence protein solubility and may also this way contribute to cataract formation.

In summary, we investigated the soluble fraction and total homogenate of non-cataractous and cataractous lenses, we found indirect evidence for the accumulation of oxidized amino acids in the non-water soluble phase of lens proteins, the presence of high molecular weight aggregates in cataractous total homogenates, and a decrease of protein concentration in the water-soluble phase of cataractous lenses. The accumulation of oxidized amino acids and the oxidative modification of Phe residues may be a link between protein damage and lens opacification.

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