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Identification of phosphorylation and acetylation sites in αA-crystallin of the eye lens (*mus musculus*) after two-dimensional gel electrophoresis

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Abstract Posttranslational modifications are of great interest because of their relevance in biological systems as proteins are commonly activated or deactivated by phosphorylation, glycation and acetylation [1, 2]. During eye lens aging the number of the αA-crystallin isoproteins increases. This could be observed by the use of 2D-PAGE (two-dimensional gel electrophoresis). The number of αA-crystallin spots in the gel increased during eye lens aging. For further analysis the spots of 2D-PAGE were cut out and the identification of the proteins was done using nanoLC-ESI-MS/MS (liquid chromatography electrospray ionization tandem mass spectrometry). The created MS/MS-data were analyzed using the Sequest algorithm. Searches with different parameters were done to preferably get the complete sequence coverage and to identify posttranslational modifications of the αA-crystallin. The acetylated N-terminus of this protein could be detected. Furthermore, phosphorylation of serine 122 and 148 was identified in two different spots.

Keywords αA-crystallin · Posttranslational modifications · NanoLC-MS/MS · Sequest

Introduction

The eye lens allows focusing images of objects on the retina. It consists of two cell types, a layer of epithelial cells and fiber cells which differentiate from the epithelial cells [3]. The lens is growing throughout life and is maintaining transparency without significant turnover of its densely packed proteins [4]. The eye lens consists of 70% proteins. Above all, the crystallins act as molecular chaperones [5]. This protein family prevents protein aggregation and in consequence the loss of eye lens transparency. The crystallins comprise more than 90% of the lens proteins, whereas the α-crystallins constitute up to 50% of the protein in the eye lens. α-Crystallins are composed of the 20-kDa subunits αA- and αB-crystallin, in a ratio of approximately 2:1 [6]. Under physiological conditions α-crystallins form loose spherical particles with an average mass of 550 kDa [7], which readily change with temperature, pH and ionic strength [8]. As a result of a very low turnover in the eye lens and the presence of the accordant enzymes, crystallins are posttranslationally modified, i.e., phosphorylated at serine 122. In vivo phosphorylation of αA-crystallin occurs predominantly at this residue [9]. Three further phosphorylation sites between amino acid 122 and 173 are previously described in the literature [10], but not yet localized to specific amino acid residues. Serine-specific phosphorylation of α-crystallins is believed to occur via cAMP-dependent protein kinase pathways [11]. The major phosphorylation of the αA-crystallin at the serine 122 appears to be an age regulated event in eye lenses [12]. An alternative pathway of phosphorylation is provided by the assertion that αA-crystallin possesses autokinase activity [13]. Conversion from oligomers to tetramers enhances this activity [14]. The N-terminus of the protein is acetylated [15] and glycation of αA-crystallin is already described in literature by Derham et al. [2].

In order to search for the described posttranslational modifications we separated the mouse lens proteins by large gel 2D electrophoresis, a high performance 2D technique leading to maximum protein resolution [16]. The spots of interest were picked out. After that, proteins were tryptically digested. The isolated peptides were extracted out of the gel and analyzed using nanoLC-ESI-MS/MS. The Sequest algorithm was used for evaluation of the produced data. Several database searches with different parameters were done to interpret the data as complete as possible.

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Experimental

Chemicals

Acetonitrile (gradient grade) and TFA were obtained from Merck, Darmstadt, Germany. Formic acid was purchased from J. T. Baker., Deventer, the Netherlands. Ammoniumhydrogencarbonate and Coomassie G-250 were obtained from Fluka, Buchs, Switzerland. Trypsin (porcine, sequencing grade modified) was obtained from Promega, Madison, USA.

Extraction of lens proteins

The procedure was described in detail by Jungblut et al. [17]. In brief, the eye lenses were taken from C57BW6 mice. Lenses were dissected from the eyes leaving the lens capsule intact, rinsed with physiological saline, freed from any adjacent fluid, weighed, and frozen immediately in liquid nitrogen. Lenses, buffer, and protease inhibitor were added to a mortar, and ground down to a fine powder. The powder was transferred into a tube, thawed, and sonicated with glass beads for homogenisation. After removal of the beads, the homogenate was mixed with urea, DTT and ampholytes (pH 2–4), and stirred for 45 min at room temperature.

2D-PAGE and sample preparation

For 2D-PAGE 4 µL of the sample was applied to a 44.5 cm IEF tube gel. Large gel 2D electrophoresis was used for separation of the proteins [16]. After the second dimension the protein spots in the gel were visualized by colloidal Coomassie G-250 staining described by Doherty et al. [18]. Spots of interest were cut out of the gel and alternately washed with digestion buffer (10 mM NH_4HCO_3, pH 7.8) and with digestion buffer / acetonitrile (1:1, v/v) for 10 min, three times each. A final washing step with acetonitrile led to shrinking of the gel piece, which was reswollen with 2 µL protease solution (trypsin at 0.05 µg/µL in digestion buffer). Digestion was performed overnight at 37 °C. The peptides were extracted from the gel with 20 µL 5% formic acid (v/v).

nanoLC-ESI-MS/MS

LC-ESI-MS/MS spectra were recorded using a Finnigan LCQclassic (ThermoQuest Finnigan, San Jose, USA) ion trap mass spectrometer equipped with a nano-electrospray ion source (PicoView 100, New Objective Inc., Woburn, USA) and distal coated SilicaTips (FS360-20-10-D, New Objective, Woburn, USA). Tryptic peptides were separated by reversed phase nano-HPLC on a 75 µm I.D.×250 mm PepMap column (Dionex LC Packings, Idstein, Germany) using the Ultimate system (Dionex LC Packings, Idstein, Germany). The flow rate was adjusted to 150 nL/min. The extracted peptides were washed on-line and pre-concentrated with 0.1% TFA at a flow rate of 30 µL/min for 10 min on a µ-precolumn (0.3 mm I.D.×1 mm, PepMap, Dionex LC Packings, Idstein, Germany). For separation the solvent system consisted of solvent A: 0.1% (v/v) formic acid and solvent B: 0.1% (v/v) formic acid / 84% (v/v) acetonitrile. The gradient was 5–15% solution B in 3 min, 15–50% solution B in 90 min and 50–95% B in 4 min. The following ESI parameters were used: spray voltage 2.3 kV, no sheath gas flow, capillary temperature 250 °C, capillary voltage 45 V, tube lens offset 30 V and the electron multiplier at –950 V. The remaining parameters of the mass spectrometer were used as described before [19].

Interpretation of the MS/MS-data

The sequences of the uninterpreted ESI-MS- and ESI-MS/MS-Spectra were identified by correlation with the NCBI-protein sequence database (http://www.ncbi.nlm.nih.gov) and two subdatabases, one containing all proteins of mouse and rat, the other of all crystallins in the NCBI using the Sequest algorithm [20, 21, 22]. For the first Sequest search the subdatabase of mouse and rat was used. The following parameters were applied: partial oxidation of methionine (+16 Da), fixed modification of cysteine with acrylamide (+71 Da), a fragment ion and peptide mass tolerance of ±1.5 Da. Furthermore, trypsin was specified as used protease. After that, the searches were repeated for the non interpreted spectra using the same parameters described above but without defining a specific protease. The next search was performed against the complete NCBI database. Subsequently, the non identified spectra were aligned once more against the subdatabase with partial phosphorylation of serine-, threonine- and tyrosine-residues (+80 Da) in addition to the parameters described above. Moreover, an additional search against the subdatabase containing all crystallins was done using a peptide tolerance of ±150 Da and a fragment tolerance of ±1.5 Da. Generally, a Xcorr value of greater than 2.0 and a dCn greater than 0.1 was accepted as a positive identification [23]. Manual inspection of each spectrum which contained modifications was performed

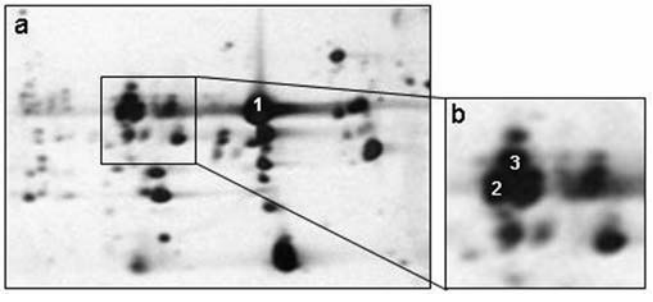

Fig. 1a, b Cutting pattern of a large 2D gel. In **a** the whole area of interest (pH 4–7, mass range 10–25 kDa) is shown. In all spots αA-crystallin was identified. The spectrum of the identified acetylation at the N-terminus was extracted of spot *1*. Moreover, the data of the table were generated using sample 1. **b** Shows an enlargement of the area in which phosphorylation sites (spot *2* and *3*) were detected

Table 1 Detected sequence coverages of spot 1 using the different search parameters. In this table the search parameters and the according sequence coverages are shown. The identified peptides are marked in bold. In the last row the summarized sequence coverage of all search results is demonstrated. The coverage increased from 75% after the first search up to 84% by addition of all search results in which peptides were identified

	Database	Stat. mod.	Diff. mod.	Protease	Pep. Tol.	Frag. Tol.	Sequence coverage	Sequence			
1	Subdatabase of mouse and rat	C+71	M+16	Trypsin	1.5	1.5	129/173, 75%	**MDVTIQHPWF** SSTISPYYR**Q** SPEDLTVKVL PSNVDQSALS **VSR**EEKPSSA	**KRALGPFYPS** **SLFR**TVLDSG EDFVEIHGK**H** CSLSADGMLT PSS	**R**LFDQFFGEG ISEVR**SDR**DK **NER**QDDHGYI FSGPKVQSGL	LFEYDLLPFL FVIFLDVKHF SR**EFHRRYRL** DAGHSER**AIP**
2	Subdatabase of mouse and rat	C+71	M+16	None	1.5	1.5	79/173, 46%	**M**DVTIQHPWF SSTISPYYRQ **SPEDLTVKVL** **PSNVDQSALS** **VSREEKPSSA**	K**RAL**GPFYPS **SLFRTVLDSG** EDFVEIHGK**H** **C**SLSADGMLT **PSS**	RLFDQFFGEG **ISEVRSDRDK** **NERQDDHGYI** FSGPK**V**QSGL	LFEYDLLPFL **FVIFLDVKHF** **SREFHRRYRL** DAGHSER**AIP**
3	Subdatabase of all crystallins		M+16, C+71	None	150	1.5	75/173, 43%	MDVTIQHPWF SSTISPYYR**Q** SPEDLTVKVL PSNVDQSALS **VSR**EEKPSSA	K**RAL**GPFYPS **SLFR**TVLDSG EDFVEIHGK**H** CSLSADGMLT PSS	RLFDQFFGEG ISEVR**SDR**DK **NER**QDDHGYI FSGPK**V**QSGL	LFEYDLLPFL FVIFLDVKHF SR**EFHRRYR**L DAGHSER**AIP**
Summary of all searches							146/173, 84%	MDVTIQHPWF SSTISPYYR**Q** SPEDLTVKVL PSNVDQSALS **VSR**EEKPSSA	K**RAL**GPFYPS **SLFR**TVLDSG EDFVEIHGK**H** CSLSADGMLTPSS PSS	RLFDQFFGEG ISEVR**SDR**DK **NER**QDDHGYI FSGPK**V**QSGL	LFEYDLLPFL FVIFLDVKHF SR**EFHRRYR**L DAGHSER**AIP**

to confirm the Sequest result. Theoretical digestions for comparison with the measured data were performed by MS-Digest (ProteinProspector, Version 3.2.1) [24].

Results and discussion

The eye lens proteins of a 14 week old mouse were separated by large 2D-PAGE. In the gel area of interest (pH 4–7, mass range 10–25 kDa) many protein spots could be visualized (Fig. 1a). In all of these spots αA-crystallin was identified using nanoLC-ESI-MS/MS and the Sequest algorithm for interpretation of the spectra. After the first search against the subdatabase of the NCBI containing all mouse and rat proteins the sequence coverage of the protein was close to or greater than 50% for all spots. The interpretation of MS/MS data is sometimes impossible using stringent search parameters because trypsin possesses some unspecific and chymotryptic side reactivity. As a consequence, nontryptic peptides occur. The database searches have to be more variable to interpret the data of these peptides. After a first stringent database search to identify the protein, a search without defining a protease must follow to identify nontryptic peptides. Furthermore, an analysis has to be done using known modifications such as acetylation or phosphorylation and moreover, a wide peptide mass tolerance to identify other modifications in the protein. The subdatabase used for the analysis of the data can be smaller if the search parameters are less stringent. In Table 1 the sequence coverages received in the different searches and the combined coverage of all database searches found in spot 1 are depicted. Several searches with the described parameters increased sequence coverage from 75% after the first search with limited parameters to 84% after additional searches with less strict parameters. The unidentified tryptic fragments of the sequence were too short to be detected using nanoLC-ESI-MS/MS if no cleavage sites were missed out. The search for phosphorylation sites in spot 1 was not successful, but in searches without defined protease non tryptic peptides were identified. Therefore, the sequence coverage was enhanced. Furthermore, by setting a wider peptide mass tolerance the identified peptides showed mass differences of explainable modifications like acetylation of the N-terminus and modification of the cysteines.

As αA-crystallin was found to be separated in different spots, we assumed posttranslational modification or protein truncation in most of these cases. Indeed, the acetylated N-terminus of the αA-crystallin was identified by Sequest database search using a wide peptide mass tolerance of ±150 Da, a fragment tolerance of 1.5 Da and without defining a specific protease. With these parameters the acetylated and partial oxidized N-terminal peptide was found in all analyzed spots of αA-crystallin. In the related spectra the fragments of the b- and y-ion series could be allocated (Fig. 2) and showed high similarity in all spots. In contrast, the C-terminus of the αA-crystallin could not be identified in any spot apart from spot 1 (Fig. 1a). A truncation of lens crystallins is described to be a common feature of both aging and cataract formation in rodents [25].

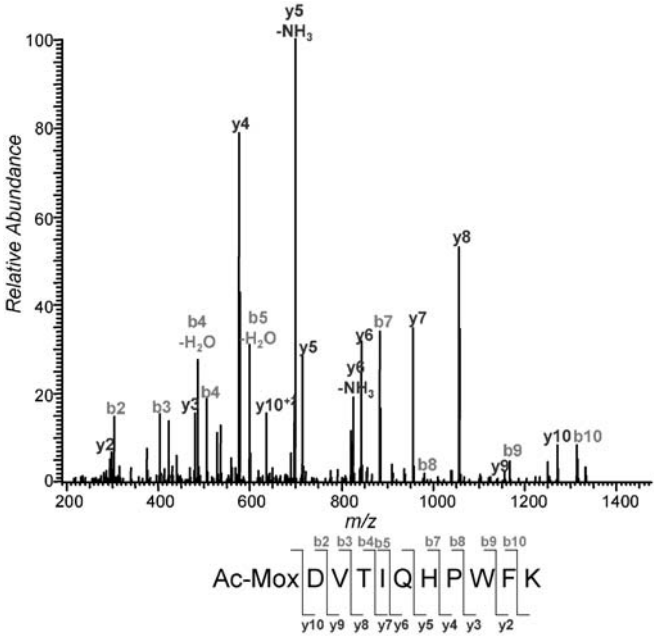

Fig. 2 The MS/MS-spectrum of the identified N-terminal peptide from spot 1. The methionine was acetylated and oxidized. Most of the b- and y-ions were identified and are marked in the spectrum. The assigned fragments are also marked in the sequence beneath

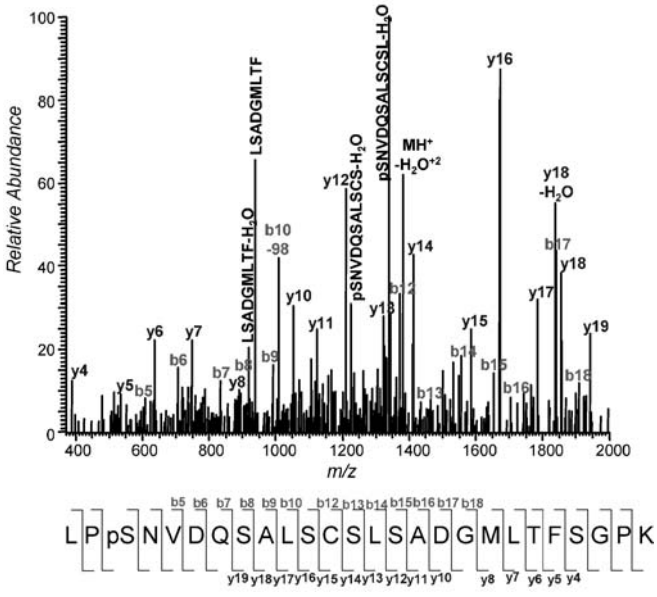

Fig. 3 The MS/MS-spectrum of the phosphorylated peptide in spot 2. Many b- and y-ions of the peptide LPpSNVDQSALSCSLSADGMLTFSGPK were identified. Furthermore, internal fragments were produced during fragmentation in the ion trap because of serine and proline residues causing preferred breaking point inside the sequence. The large ones were marked, smaller fragments were found in the background peaks of the spectrum

Moreover, two phosphorylation sites could be identified. αA-crystallin forms each with only one phos-

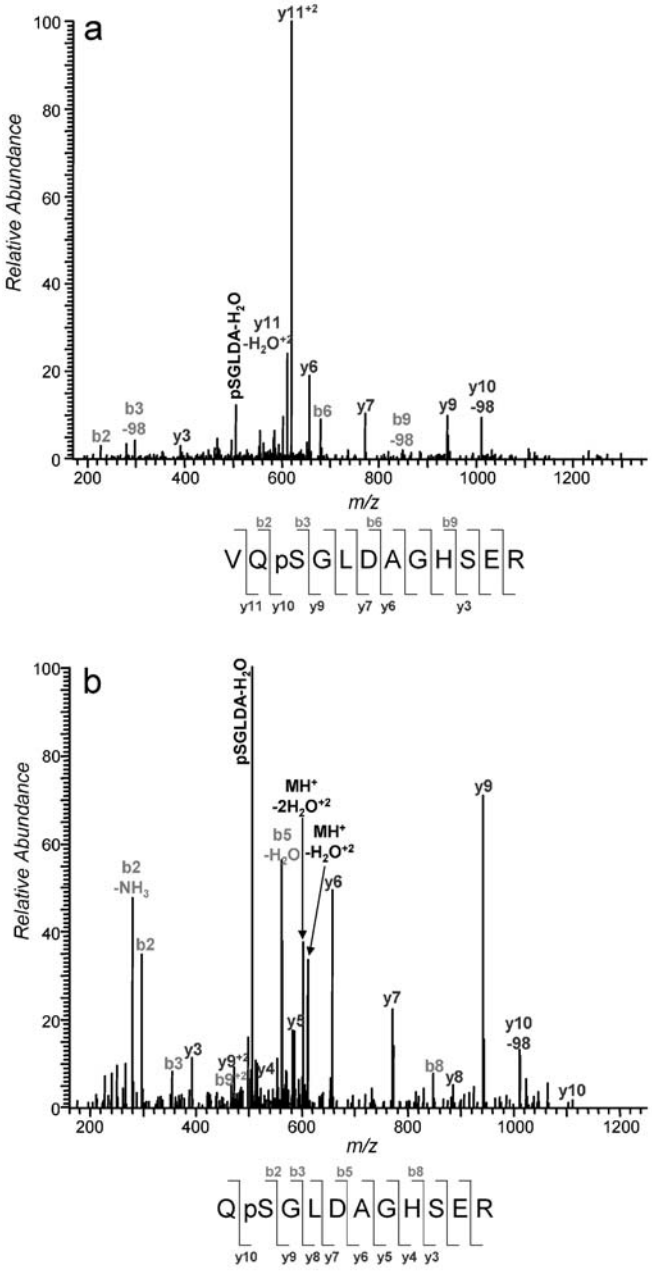

Fig. 4a, b MS/MS-spectra of the phosphorylated peptide in spot 3. In **a** the spectrum of the phosphorylated tryptic peptide VQSGLDAGHSER is shown. Most of the y-ions, but only a few b-ions were allocated. The phosphorylation of the ions was demerged in part. In **b** the spectrum of the phosphorylated non tryptic peptide QSGLDAGHSER is demonstrated. The complete y-ions from y3 to y10 were allocated. Some of the corresponding b-ions were also identified

phorylation at a time were separated in adjacent, but separated spots in the gel (Fig. 1b). Figure 3 shows the MS/MS-spectrum of the phosphorylated peptide in spot 2. In spot 3 a phosphorylation site could be identified at serine 148 in the tryptic peptide from amino acid 146 to 157 [VQSGLDAGHSER]. In this fragment ion spectrum, ions of the b- and y-series were detected (Fig. 4a). Furthermore, a non tryptic peptide similar to this peptide, but without valine at the N-terminus was identified also within the same nanoLC-MS/MS run (Fig. 4b). No direct description of this site could be found in literature, but it is annotated that there are three phosphorylation sites between serine 122 and the C-terminus of αA-crystallin identified in peptide mapping experiments [9]. In spot 2 a second phosphorylation site at serine 122 was observed. This is a prominent site for phosphorylation in αA-crystallin described earlier [26, 27, 28, 29, 30, 31, 32, 33]. The interpretation of the appendant spectrum is difficult because of the numerous serine and proline residues in the primary structure of the tryptic peptide [LPpSNVDQSALSCSLSADGMLTFSGPK]. These residues affect the occurrence of many internal fragments. Nevertheless, the interpretation of the fragment spectrum can explain most of the large peaks with ions of the b- or y-series (Fig. 3). Remaining large peaks are explainable upon formation of internal fragments. The smallest identified fragment ions are the b5-ion and the y4-ion. Peptide fragments with lower masses could not be detected. Most of the smaller peaks in the spectrum are resulting from internal fragments or fragments after elimination or splitting of water or of the phosphorylation group. The phosphorylation of serine 122 appeared to be an age regulated event in eye lenses [32]. Both identified phosphorylation sites are potential targets of cAMP-dependent kinase with a (R/K)-(X_{1-2})-S recognition site [9]. Indeed, the localized phosphorylation sites were not found in one single spot at the same time. This leads to the assumption that only one phosphorylation at a time might be possible.

The identified phosphorylation sites confirm the data of Ueda et al. [25]. In this publication the PAWS software was used for determination of the truncation site. Furthermore, the isoelectric point of the identified truncated species was calculated using the GeneWorks software. The measured isoelectric points of the partially degraded α-crystallins were found to match their calculated isoelectric points. An exception was the αA-crystallin fragment 1–165, that was expected to migrate to a pI nearly identical to the intact protein, but migrated to a more acidic point. The authors could not give an explanation concerning this shift. The present study might explain this pH shift as αA-crystallin is phosphorylated at one serine residue. The described phosphorylations affect the more acidic character of the protein leading to a more acidic isoelectric point.

The present results are in line to the data published by Ueda et al. [25] as shown in Table 2. Ueda and colleagues showed that many of the different detected spots in the gel were explainable by truncation of the C-terminus. In contrast, MacCoss et al. published the localization of different posttranslational modifications in the αA-crystallin of human lens tissue using the multidimensional protein identification technology (MudPIT) [32]. The identification of four phosphorylation sites at serine or threonine residues, three oxidation sites of tyrosine or methionine residues, the acetylation of four lysine residues and the methylation of one arginine and one lysine residue were described. Only one of these modifications, the phosphorylation of serine 122, could be identified in our work. However, the acety-

Table 2 Summary of the presented data. Data of the described literature are compared and the newly presented data are printed in bold. The new data complements the data of earlier literature

	Chiesa et al. [9]	Ueda et al. [25]	MacCoss et al. [32]	Present data
Tissue	Bovine lenses	Rat lenses	Human cataract lenses	Mouse lenses
Protein description (Swiss-Prot)	sp|P02470|CRAA_BOVIN Alpha crystallin A chain – Bos taurus (Bovine)	sp|P02490|CRAA_MOUSE Alpha crystallin A chain, major component – Rattus norvegicus (Rat)	sp|P02489|CRAA_HUMAN Alpha crystallin A chain – Homo sapiens (Human)	sp|P02490|CRAA_MOUSE Alpha crystallin A chain, major component – Mus musculus (Mouse)
Primary protein sequence	MDIAIQHPWF KRTLGPFYPS RLFDQFFGEG LFEYDLLPFL SSTISPYYRQ SLFRTVLDSG ISEVRSDRDK FVIFLDVKHF SPEDLTVKVQ EDFVEIHGKH NERQDDHGYI SREFHRRYRL PSNVDQSALS CSLSADGMLT FSGPKIPSGV DAGHSERAIP VSREEKPSSA PSS	MDVTIQHPWF KRALGPFYPS RLFDQFFGEG LFEYDLLPFL SSTISPYYRQ SLFRTVLDSG ISEVRSDRDK FVIFLDVKHF SPEDLTVKVL EDFVEIHGKH NERQDDHGYI SREFHRRYRL PSNVDQSALS CSLSADGMLT FSGPKVQSGL DAGHSERAIP VSREEKPSSA PSS	MDVTIQHPWF KRALGPFYPS RLFDQFFGEG LFEYDLLPFL SSTISPYYRQ SLFRTVLDSG ISEVRSDRDK FVIFLDVKHF SPEDLTVKVQ EDFVEIHGKH NERQDDHGYI SREFHRRYRL PSNVDQSALS CSLSADGMLT FCGPKIQTGL DAGHSERAIP VSREEKPTSA PSS	MDVTIQHPWF KRALGPFYPS RLFDQFFGEG LFEYDLLPFL SSTISPYYRQ SLFRTVLDSG ISEVRSDRDK FVIFLDVKHF SPEDLTVKVL EDFVEIHGKH NERQDDHGYI SREFHRRYRL PSNVDQSALS CSLSADGMLT FSGPKVQSGL DAGHSERAIP VSREEKPSSA PSS
Amino acids	173 AA	173 AA	173 AA	173 AA
Molecular weight	19790 Da	19792 Da	19909 Da	19792 Da
Isoelectric point	5.8	5.8	5.8	5.8
Method	Peptide mapping experiments	2D-PAGE, mass spectrometry, assignment of protein cleavage site and calculation of isoelectric points	Tryptic digestion, Multidimensional protein identification technology (MudPIT)	2D-PAGE, tryptic digestion and mass spectrometry, Sequest database search
Phosphorylation sites	S122, three sites between S122 and S173, not localized	No results are presented	T13, S45, S122, T140	S122, **S148**
Acetylation sites	No results are presented	No results are presented	K70, K78, K88, K145	**M1**
C-Terminus	No results are presented	The C-terminus could be determined in single spots. The acidic shift of one spot could not be explained	No results are presented	The C-terminus could be determined in one spot. **The acidic shift of two spots could be explained by phosphorylation of the αA-crystallin**

lation of the N-terminal methionine could be identified in all analyzed spots in the present work. This modification site was not described by MacCoss et al. Therefore, our results are not in line with the results of MacCoss. Hence, a confirmation of the published data has to be done in further studies.

Conclusion

After separation of mouse eye lens proteins by large 2D-PAGE, spots of the related gel region were analyzed after tryptic digestion. The αA-crystallin was identified with high sequence coverages in all these spots. Furthermore, the phosphorylation of serine 122 and serine 148 was observed. These sites were described in literature but were not identified using 2D-PAGE and nanoLC-MS/MS so far. The N-terminus of αA-crystallin is quantitatively acetylated. These results agree with the descriptions in literature. Furthermore, the acidic shift of some spots described earlier in literature [25] is now explainable by phosphorylation of one serine residue in the protein. Altogether αA-crystallin was identified with comprehensive sequence coverage, and the identification of posttranslational modifications was possible.

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