Multiple Separations Facilitate Identification of Protein Variants by Mass Spectrometry

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Multiple separations facilitate identification of protein variants by mass spectrometry

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Identification of variant proteins from complex biological samples promises to contribute much to our understanding of the etiology of pathological states. Characterization of variants, due either to genetic mutations in protein sequences or to post-translational modifications, is considerably more difficult than the simple protein identifications typical of most current proteomic investigations. Identification of a few peptides by database retrieval is not adequate when the goal is to have a complete understanding of the modifications of the protein. Although one advantage of mass spectrometry is its ability to obtain specific responses to several components, the complexity of biological samples is often overwhelming, resulting in spectra lacking useful information. For complex mixtures, isolation procedures before mass spectrometric analysis may need to include a variety of chromatographic and electrophoretic separation techniques. In this report, we illustrate how several preparative steps were essential for obtaining information about modified human lens β-crystallins. The preparative techniques prior to mass spectrometry included size-exclusion chromatography, reversed-phase chromatography, two-dimensional gel electrophoresis, in situ digestion of the proteins and peptide trapping and washing before a final reversed-phase high-performance liquid chromatographic separation on-line to the mass spectrometer. This approach for isolation and analysis, when customized for other proteins, should find application in many studies where protein variants of complex mixtures are to be identified.

Keywords: chromatography, gel electrophoresis, HPLC/electrospray ionization mass spectrometry, lens β-crystallins, tandem mass spectrometry

Introduction

The primary goal of most proteomic endeavors has been to identify proteins separated by two-dimensional gels, with particular attention to those found in pathological conditions. In this mission, a common approach is to enzymatically digest the protein in situ, elute the peptides and identify several peptides by matching their molecular masses, determined by mass spectrometry, with expected molecular masses of peptides from proteins with known sequences. For positive identification, the fragmentation patterns from analysis by tandem mass spectrometry (MS/MS) can be used to confirm the sequence. Success in identifying a protein often requires identification of only a few peptides.

However, simple identification of the protein is not sufficient for an understanding of the role of proteins in disease states. For proteomics to reach its potential in pathology, protein variants, both genetic variants in the amino acid sequences and post-translational modifications, will need to be identified. It will not be sufficient to identify a few peptides eluted from a gel by matching masses with protein databases. Peptides from all portions of the protein sequence must be retrieved from the gel and their masses and sequences determined. In addition, in making these identifications, the presence of peptides from more than one protein in the same digest is likely to cause confusion. Although the ability to analyze mixtures is one of the major advantages of mass spectrometry, complete analysis of proteins present in complex mixtures often requires one or more steps of separation prior to analysis. Both chromatography and electrophoresis have been used extensively to purify proteins prior to analysis by mass spectrometry. This contribution demonstrates the advantage of combining several isolation techniques prior to mass spectrometry to maximize information about proteins in mixtures.

The proteins of the human lens, called crystallins, provide an example of the complexity of proteins in biological samples. Although the human lens has only eleven gene
products, the crystallins, which do not undergo turnover, become extensively modified with aging, leading to a complex mixture of proteins in older lenses. Identification of these modified proteins is of particular interest because they are likely the precursors of cataract, the principal cause of blindness worldwide. Because of their high sequence homology and extensive modification, the crystallins are difficult to separate. In our studies, we have found that neither chromatography nor two-dimensional gel electrophoresis alone provides adequate separation for mass spectrometric analysis. The similar hydrophobicity of some crystallins permits only partial separation by reversed-phase high-performance liquid chromatography (HPLC) and, likewise, the similar pI values and charge changes of some of the modifications prevent adequate separation by ion exchange. Even with dual separation by molecular mass and pI in two-dimensional gel electrophoresis, some proteins with very different sequences migrate to the same position. We have found that a combination of several types of chromatography and electrophoresis, taking advantage of the unique separating powers of each, permits sufficient separation for mass spectrometric analysis of the entire sequence of the protein. The procedure includes several chromatographic steps before two-dimensional gel separation, followed by elution of proteins either intact or as peptides after enzymatic digestion, with subsequent trapping and washing to remove components that would interfere with mass spectrometric analysis. Determination of the molecular masses of the proteins and peptides by on-line capillary HPLC/MS with further analysis by MS/MS then permits complete identification of all the protein modifications. With appropriate variations, procedures similar to this method, which was developed specifically for lens β-crystallins, may find application for other proteins.

Materials and methods

Chemicals

Bromophenol blue, dithiothreitol (DTT), CHAPS, pH 3–10 non-linear (NL) Immobiline DryStrips, pH 3–10 NL immobilized pH gradient (IPG) buffer and DryStrip cover fluid were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Urea was from Gallard–Schlesinger Industries (Carle Place, NY, USA). HPLC-grade acetonitrile was from EM Science (Gibbstown, NJ, USA). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI, USA). All other chemicals were from Sigma Chemical Co. (St Louis, MO, USA).

Preparation of water-soluble human lens crystallins

Normal transparent lenses, ages 2, 55, 56 and 59 were obtained from the Lions Eye Bank, Omaha, NE, USA. The donor history for each clear lens did not indicate any diseases associated with cataracts. Each lens was individually homogenized using a stirring bar for three hours in 2.5 mL of 50 mM MES buffer containing 0.9% NaCl, 1 mM EDTA and 1 mM DTT (pH 6.0) at 4°C. After centrifugation at 15,000 g for one hour, the water-soluble and water-insoluble crystallins were separated.

Size-exclusion chromatography of water-soluble human lens crystallins

The water-soluble crystallins were separated into α-, β- and γ-crystallins by size-exclusion chromatography using a Superose 6 HR column (Amersham Pharmacia Biotech) and a solvent delivery system (Dynamax, Rainin, Woburn, MA, USA). The separation was performed isocratically with the same buffer that was used for homogenization at a flow rate of 0.4 mL min⁻¹. Protein elution was monitored at 280 nm. All the β-crystallins were collected in one fraction and used in the next step of isolation.

Reversed-phase HPLC separation of water-soluble β-crystallins

The β-crystallins isolated by size-exclusion chromatography were fractionated by reversed-phase HPLC. The concentration of β-crystallins was estimated by a bicinchoninic acid assay (Pierce, Rockford, IL, USA). Before the reversed-phase HPLC separation, the β-crystallins were denatured in 6 M guanidine hydrochloride and 100 mM DTT at room temperature overnight, acidified to pH 4.0 by gradual addition of dilute trifluoroacetic acid (TFA) and concentrated by ultrafiltration (Centriprep 10) with a 10 kDa cut-off (Millipore, Bedford, MA, USA). The resulting solution was centrifuged at 14,000 g for 10 min to remove water-insoluble particles. About 200 μg of β-crystallins were fractionated by reversed-phase chromatography (4.6 × 150 mm C4 column, 300 Å, 5 μ, Vydac, Hesperia, CA, USA). The proteins were eluted using 0.1% TFA in H₂O (solvent A) and 0.1% TFA in 95% acetonitrile (solvent B) and a gradient starting with 25% solvent B for 5 min, increasing from 25 to 60% in 35 min and from 60 to 98% in 3 min. The βB1 and βA4 fractions from each run were pooled and stored at –80°C.

Anion-exchange chromatographic separation of the proteins in the βB1 and βA4 fractions obtained from reversed-phase HPLC

The βB1 and βA4 fractions collected from reversed-phase HPLC were evaporated to a dry powder and re-suspended in a weak mobile phase containing 20 mM piperazine, 6 M urea and 0.1% DTT (pH 9.5) and incubated at room temperature for two to three hours. The anion-exchange separation used a Mono Q anion-exchange column (Amersham Pharmacia Biotech). The strong mobile phase was the same buffer, but containing 0.5 M NaCl. The gradient was 0–20% in 5 min, and 20–50% in 40 min.
were determined using a single quadrupole electrospray ionization (ESI) mass spectrometer (Micromass Platform II, Manchester, UK). After desalting and urea removal, the proteins in the anion-exchange fractions were further fractionated by on-line LC/MS using a microbore column (1.0 × 50 mm C4, 300 Å, 5 μ, MicroTech, Sunnyvale, CA, USA) with a gradient of 25–55% of acetonitrile with 0.1% TFA in 30 min at a flow rate of 50 μL min⁻¹. A post-column splitter (Upchurch Scientific, Oak Harbor, WA, USA) divided the flow into two portions with 10% of the flow entering the mass spectrometer and 90% collected and saved for other experiments.

Two-dimensional gel electrophoresis of proteins in βB1 and βA4 fractions obtained from reversed-phase HPLC

The βB1 and βA4 fractions collected from reversed-phase HPLC (200 μg per sample) were first lyophilized using a speed vacuum concentrator (Savant SC 110, Farmingdale, NY, USA) and then re-dissolved in a buffer containing 8 M urea, 2% CHAPS, 2% pH 3–10 NL (nonlinear) buffer and 20 mM DTT. To eliminate precipitation of the sample and increase the loading capacity, the sample was loaded by rehydration, in which the sample solution was absorbed by the dry IPG gel strips. The strips (13 cm) containing protein samples were placed on the cooling plate of a Multiphor II system (Amersham Pharmacia Biotech) and covered with mineral oil to prevent oxidation. The first-dimensional isoelectric focusing (IEF) gel electrophoresis was performed at 150 V, 300 V and 600 V for one hour in each step followed by 3500 V overnight (total Vh 53,550). The temperature was kept constant at 30°C using a Lauda Ecoline water circulation system (Dinksmann, Germany).

After the IEF was finished, each IPG strip was equilibrated with a buffer containing 6 M urea and 30% (w / v) glycerol, 2% (w / v) sodium dodecyl sulfate (SDS) and 1% (w / v) DTT for 15 min. In the second equilibration, the strips were incubated in a buffer containing 4% (w / v) iodoacetamide for another 15 min. The strips were then placed on the top of pre-cast 15% T and 2.6% C SDS-polyacrylamide gel electrophoresis (PAGE) gel, and fixed with 0.5% acetic acid. The second SDS-PAGE gel electrophoresis was performed at 100 V overnight using a Hoffer SE 400 vertical SDS-PAGE gel electrophoresis apparatus system (Amersham Pharmacia Biotech) with the temperature maintained at 4°C. After the electrophoresis was completed, proteins on the SDS-PAGE gel were visualized by Coomassie blue staining. Coomassie blue staining is very reproducible but has a limit of detection of about 0.1–0.5 μg. The intensities of the spots usually show a linear relationship to quantity of sample (up to 20 μg cm⁻²). For visualization of proteins that were less abundant, negative zinc acetate staining was used because it has a limit of detection of about 5 ng. Another advantage of negative zinc acetate staining is the short staining time (40 to 45 min), which minimizes the chance for artificial protein modifications.

Identification of proteins in the spots of interest using electro-elution and on-line LC-ESI mass spectrometry

Before mass spectrometric analysis, the SDS was removed from the samples, and the proteins were desalted and concentrated by passing the sample through both an SDS trap and a protein trap cartridge (Micromass BioResources, Auburn, CA, USA) connected to a 10-port Valco injection valve (Alltech, Deerfield, IL, USA). After elution from the gel in a basic buffer (0.1% SDS, 25 mM Tris, 0.2 M glycine, pH 8.6), the sample was acidified to pH 5.0–6.0 by addition of dilute TFA and loaded into the SDS removal cartridge with the injector in the load position. The proteins were eluted from the SDS trap and delivered to the protein trap, where the proteins bind to the solid phase, by using 500 μL of a solution of 2% acetonitrile containing 0.05% TFA. The valve was then turned to put the protein trap in line with the capillary column (0.3 × 50 mm C4, 300 Å, 5 μL LC-Packings, San Francisco, CA, USA) and the proteins eluted from the trap onto the column. Meanwhile, the SDS trap, which was now isolated from the protein trap, was regenerated by backflushing with 90% acetonitrile containing 0.1% HCl. A solvent delivery system (Shimadzu LC/10 AD VP, Shimadzu Scientific Instrument, Columbia, MD, USA) and control (Acurate Microflow Processor, LC-100-VAR, LC-Packings) maintained a flow of 5 μL min⁻¹ for LC-MS. A binary system of 0.05% TFA in H₂O (solvent A) and 0.05% TFA in 95% acetonitrile (solvent B) and a gradient of 25% solvent B for 5 min, 25–50% in 40 min and 50–98% in 5 min was used. Protein elution was monitored by a capillary Z-shaped U-Z View flow cell (8 mm–path length, 35 mL volume, LC-Packings). The eluted proteins were identified by their molecular masses determined by a single quadrupole ESI mass spectrometer (Micromass Platform II).

In-situ trypsic digestion of the spots containing the β-crystallins of interest

The spots containing β-crystallins were excized from the negative zinc acetate stained gel and the proteins in the spots were digested with trypsin in situ according to the procedure of Courchesna and Patterson with minor modifications. The excized gel spots were incubated in 50 mM citric acid at room temperature for 20 min to mobilize the protein in the gel matrix, followed by two more incubations, each for 30 min in 20 mM NH₄HCO₃, 50% acetonitrile. The gel slices were then lyophilized to dryness. To perform the in-gel trypsinic digestion, the gel slices were placed in a mixture of 1 μL of 0.1 μg μL⁻¹ sequence-grade modified trypsin (Promega) and 15 μL of 20 mM NH₄HCO₃ in 1 mM CaCl₂. This step was repeated until the gel slices would absorb no more solution. Additional buffer, equal to three times the volume of trypsin solution absorbed, was added and the gel slices were incubated at 37°C overnight. The next day, the supernatant was removed and saved. The digested peptides were extracted from the gel matrix by rotation at 37°C for
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30 min in 200 µL of 1% TFA in 60% acetonitrile and by additional rotation in 200 µL of 0.1% TFA in 60% acetonitrile under the same conditions as described above. All three supernatants were combined and concentrated to 50 µL by lyophilization.

Characterization of modifications of β-crystallin peptides by on-line LC/MS and collision-induced dissociation (CID) tandem mass spectrometry (MS/MS)

The peptides extracted after in situ digestion were analyzed by on-line HPLC-ESI-MS using a capillary column (C₁₈, Pepmap, 0.3 × 250 mm, LC-Packings) and an ion trap ESI mass spectrometer (LCQ, Finnigan, San Jose, CA, USA). On-line desalting used a peptide-trap cartridge (Michrom BioResources) and a controlled solvent delivery system described previously. For peptide elution, a binary system of 0.01% TFA in H₂O (solvent A) and 0.01% TFA in 95% acetonitrile (solvent B) was used. The gradient was 2% solvent B for 5 min, 2–50% in 70 min and 50–98% in 3 min. The eluted peptides were monitored at 214 nm.

Mass spectrometric analysis of the in situ tryptic peptides used the dynamic-exclusion double-play option. The data-dependent scans, including a full scan and subsequent CID and MS/MS scan were repeated three times within 30 seconds. The exclusion duration was 2 min and the exclusion mass width was 1.0 mass unit. Regular double play, including a full scan (profile mode) followed by CID MS/MS of selected parent ions was used for characterization of modifications of β-crystallins. The collision energy was 40%, as defined by the LCQ software, and the isolation window was 2.5 mass units.

Results and discussion

To illustrate the problems that may be encountered in determining post-translational modifications of biological samples, we have chosen human lens crystallins, in particular the β-crystallins. Unmodified human lens crystallins have molecular masses of approximately 20–30 kDa. They were originally named according to the size of aggregates they form in solution and their elution pattern when separated by size-exclusion chromatography (Figure 1). The first peak, called α-crystallins, is composed of aggregates of about 800 kDa (40 monomers per aggregate), the second, third and fourth peaks contain differing ratios of the same six proteins (β-crystallins) and are aggregates of 80–160 kDa, whereas the fifth peak is the monomeric γ-crystallins. The last peak contains low molecular weight (LMW) truncated proteins of less than 20 kDa. This simple chromatographic procedure is usually the initial step in simplifying crystallin mixtures for analysis. The primary difference between size-exclusion chromatograms (Figure 1) for crystallins from a 2-year-old and a 55-year-old lens is that some of the α-crystallins elute as higher molecular aggregates (HMW) in the older lens.

![Figure 1. Chromatograms showing the size-exclusion separation of water-soluble lens crystallins from a 2-year-old lens (top) and a 55-year-old lens (bottom). The β-crystallin fractions were collected, combined and used for further analyses.](image-url)

The human lens β-crystallins, which include three acidic subunits, βA1, βA3 and βA4, and three basic subunits, βB1, βB2 and βB3, offer a good illustration of the advantages of multiple separation techniques in analyzing post-translational modifications. Because of the high homology of the β-crystallins and their many age-related modifications, β-crystallins from adult lenses are mixtures so complex that no one electrophoretic or chromatographic procedure yields samples sufficiently simple for mass spectrometric analysis. In a previous publication using two-dimensional electrophoresis, co-migration of some crystallins to the same position was evident. Although in situ tryptic digestion of the proteins and analysis of the peptides by mass spectrometry identified several modified β-crystallins, the isolation was not adequate for determining the nature and location of all the modifications. Similarly, the peaks obtained after reversed-phase HPLC fractionation of the β-crystallins from adult lenses are complex mixtures. The reversed-phase HPLC chromatograms of the β-crystallins from a 2-year-old and a 55-year-old lens (Figure 2) show the βA1/A3 peak becoming smaller and broader and the peaks attributed to βB1 and βA4 merging as the lens ages. Also, a large, broad late-eluting peak appears. The complexity of the mixture of proteins in the βB1 and βA4 HPLC peak permitted only partial identification of the modifications by mass spectrometry. Because of the difficulties in analyzing this peak, we have chosen the merged βB1 and βA4 peak to illustrate, in this report, the advantages of using multiple chromatographic and electrophoretic steps before mass spectrometric analysis.

Before deciding on the use of multiple isolation steps, a variety of alterations in the conditions for the reversed-phase HPLC were explored in attempts to improve the resolution of the β-crystallins. These alterations included use of shal-
low gradients as low as 0.2% per minute, changing the organic modifier to a mixture of 50:50 acetonitrile + isopropanol, changing the ion-pairing reagent to heptafluorobutyric acid or formic acid and using a column jacket with a temperature of 50°C. The effectiveness of pre-treating the proteins with 6 M guanidine hydrochloride and 100 mM DTT for several hours was also evaluated. None of these variations resulted in significant improvement in the HPLC resolution of the β-crystallins.

For many applications, ion exchange is effective for separating complex mixtures. Some success in separating bovine β-crystallins by anion exchange has been reported. However, for the human β-crystallins in the βB1 and βA4 peaks, ion exchange did not prove useful. The anion-exchange chromatogram (Figure 3) shows four principal peaks followed by a poorly-resolved broad fraction apparently containing several components. The crystallins in each fraction were further separated by reversed-phase HPLC and analyzed by mass spectrometry. The reversed-phase HPLC chromatogram of the first peak from the anion exchange is shown in the inset of Figure 3. This HPLC chromatogram indicates the presence of as many different proteins as before the anion exchange (Figure 2). The protein in fraction A was βB2, fraction B contained βA3 (residues 23–215) and βB2 and both fractions C and D contained mixtures of βB2, intact βB1, βB1(16–215), βB1(40–215) and βB1(41–215). These results confirmed the previously reported N-terminal cleavage of βA3 and βB1. The mass spectrometric analysis showed that peak 4 of the anion-exchange also contained intact βB2, but the proteins in other anion exchange fractions could not be identified due to the complexity of the mass spectra. It appears that the various β-crystallins have been extensively modified so that they co-elute on ion-exchange chromatography, preventing successful separation.

Data from two-dimensional electrophoresis of the HPLC peak containing βB1 and βA4 (Figure 4) demonstrated why the above techniques were not successful in producing samples that could be analyzed by mass spectrometry. The 2-year-old lens has only seven major proteins in this HPLC peak whereas the 55-year-old lens has over twenty proteins with various pI values and molecular masses. To obtain a more detailed analysis of the peak attributed to βB1 and βA4, each spot of the two-dimensional gel was digested with trypsin in situ and the peptides were eluted and analyzed by mass spectrometry. From this analysis, it was evident that this peak contained not only βB1 and βA4, but also some βB2 and βA3.
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Mass spectra of intact proteins eluted from gels give useful information about the number and nature of the post-translational modifications, but are more difficult to obtain than mass spectra of peptides after digestion of the protein. The poor mass spectra of the proteins may be due to low efficiency in eluting the proteins and/or difficulties in removing other components that interfere with the mass spectral response of the proteins. For the β-crystallins from older lenses, we were able to obtain useful spectra only for intact PB2 eluted from several spots. Even when peptide analysis indicated that the spot contained several proteins, PB2 was often the only recognizable mass. This may be because the mass spectral response of PB2 is better than that of other β-crystallins or because PB2 is present with very little modification whereas the other β-crystallins are present in many forms, including a variety of truncations.

To illustrate the approach used to determine the post-translational modifications of β-crystallins, we have chosen spot 13 of Figure 4. This position corresponds to spot 14 in a previous publication in which the total proteins from two lenses, a 54- and a 55-year-old, were analyzed by two-dimensional electrophoresis without prior separation by size-exclusion and reversed-phase HPLC. In that study, several peptides from βA4, βA3 and γS were recognized after in situ trypsin digestion and mass spectrometric analysis. Some masses suggested the presence of deamidation, but the signal-to-noise ratio was not adequate to locate the deamidations with certainty. In the present study, βA4 and βA3 (γS had been removed by size-exclusion chromatography) were confirmed as components of spot 13. Inclusion of the additional chromatographic steps improved the recovery of βA4 and βA3 so that peptides from all portions of the sequences were detected. The only peptides not detected were those with masses < 300 Da, the lower limit of the mass range being scanned. The signal-to-noise ratios of the mass spectra were adequate for determining the sites of deamidation as well as an estimate of the extent of modification from the isotopic patterns. With this procedure, the digest of spot 13 yielded peptides showing five sites of deamidation, three truncation sites and three oxidized methionines in βA3, as well as a deamidation, a truncation, an oxidized methionine and an oxidized tryptophan in βA4.

To determine whether these modifications may have occurred during isolation, the lens from a 2-year-old was analyzed by the same procedure. No evidence for these modifications was found, indicating that they were in vivo modifications associated with aging.

Figure 4. Coomassie blue-stained IPG two-dimensional gel of the proteins that eluted in the HPLC peak attributed to βB1 and βA4 (see Figure 2) from a 2-year-old lens (top) and a 55-year-old lens (bottom).

Figure 5. On-line LC-ESI-MS analysis of peptides from in-gel trypsin digestion of spot 13 (Figure 4). (a) Chromatogram of the reversed-phase HPLC separation of the peptides. (b) Mass spectrum of the peptide eluting at 5.30 min. The peak at m/z 604.9 is due to the doubly-charged ion of peptide 55-64, indicating cleavage of βA3 between Asn 54 and Val 55. (c) Mass spectrum of the peptide eluting at 11.02 min. The peak at m/z 687.5 is the singly-charged ion of peptide 27-32 of βA3, indicating cleavage of βA3 between Gly 26 and Ser 27. (d) Mass spectrum of the peptide eluting at 12.36 min. The peak at m/z 1039.7 is the singly-charged ion of peptide 23-32 of βA3, indicating cleavage of βA3 between Asn 22 and Pro 23.
Figure 6. CID MS/MS spectrum corresponding to the mass spectrum in Figure 5(c). The sequence of βA3 peptide 27–32 and the expected fragments are shown diagrammatically at the top. Evidence of the corresponding fragments in the spectrum confirmed identification of this peptide as βA3 27–32. This peptide, which would not be produced by trypsin cleavage, indicates an in vivo cleavage site in βA3 between Gly 26 and Ser 27.

Part of the on-line, reversed-phase HPLC chromatogram of tryptic peptides from spot 13 is shown in Figure 5(a). The mass spectra of three peptides (their elution times are marked) illustrating truncation of βA3 are shown in Figures 5(b), 5(c), and 5(d). The peak at m/z 604.9 in Figure 5(b) is the doubly-charged ion of peptide 55–64 of βA3, which elutes at 5.30 min. It is a very minor component and, without pre-separation of β-crystallins by reversed-phase HPLC and separation of the βB1 and βA4 fraction by two-dimensional electrophoresis, it would not have been detected. The presence of this tryptic peptide indicates there has been cleavage between Asn 54 and Val 55. Because Asn 54 is one of the deamidated residues of βA3, the presence of peptide 55–64 suggests that cleavage occurred after deamidation via the mechanism demonstrated for Asp 101 of αA-crystallins. The peak at m/z 687.5 in Figure 5(c) is the singly-charged ion of peptide 27–32 of βA3, which elutes at 11.02 min [Figure 5(a)]. Because Gly 26 / Ser 27 is not a tryptic cleavage site, the presence of this peptide indicates in vivo cleavage of βA3. The peak at m/z 1039.7 in Figure 5(d) is the singly-charged ion of peptide 23–32, eluting at 12.36 min and corresponding to the previously reported N-terminal cleavage of βA3 between Asn 22 and Pro 23. The in vivo cleavages in βA3 at Gly 26 / Ser 27 and Asn 54 / Val 55 have not been previously reported.

The proteins in spot 13 were identified by LC-ESI-MS using both the dynamic-exclusion double-play mode and the general double-play mode. In the dynamic-exclusion double-play mode, the peptide with the highest intensity from the first full survey scan (centroid mode, 300 to 2000 m/z) is chosen as the parent ion. In the following CID MS/MS scan, this selected parent ion is fragmented into smaller daughter ions. This two-step cycle was repeated three times within 30 s (a typical peak width for peptides in a capillary HPLC separation) to obtain adequate signal for an MS/MS spectrum of good quality. After that, the selected parent ion was excluded and the second parent ion was selected for MS/MS analysis, even though the previously chosen ion was still the strongest ion in the scan at that time. Compared with a general double-play operational mode, more peptide ions can be selected during one run and thus more sequence information can be obtained. The general double-play (profile) mode demonstrates the isotopic envelope of peptides, which can help confirm the identities of peptides by their charge states. This operational mode is quite useful in characterizing some modifications of peptides, such as deamidation. The combi-
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Figure 7. Diagram of the multiple steps used to identify human lens β-crystallins and their modifications. The examples are from the βB1 and βA4 peak of the reversed-phase fractionation.

In-gel tryptic digestion

Reversed phase HPLC

Anion exchange HPLC

Ion trap ESI-MS/MS

CID MS/MS

Figure 7. Diagram of the multiple steps used to identify human lens β-crystallins and their modifications. The examples are from the βB1 and βA4 peak of the reversed-phase fractionation.

Concluding remarks

A summary of the procedure used in this examination of β-crystallins is given in Figure 7. For these proteins, prior size-exclusion chromatography and reversed-phase HPLC reduced the number of components per spot in the two-dimensional electrophoresis gels and improved the signal-to-noise ratio in the subsequent mass spectrometric analyses. The recovery of peptides from in-gel digestion was as effective as would be obtained from test tube digestion. Although anion-exchange chromatography was not useful for β-crystallins, inclusion of an ion-exchange step may be effective for other protein mixtures. Improved ESI mass spectra were obtained by including traps to remove chemicals used in the gel separations as well as by reversed-phase HPLC immediately before mass spectral analysis. This type of procedure, with appropriate variations, will find application in identifying the modifications of proteins from many complex biological samples.

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References


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