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Simultaneous Reduction and Alkylation of Protein Disulfides in a Centrifugal Ultrafiltration Device Prior to Two-Dimensional Gel Electrophoresis

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Abstract: Reduction and alkylation of protein disulfides prior to IEF, when performed directly in a centrifugal ultrafiltration device, provides an effective means of terminating the alkylation reaction, concentrating the proteins for analysis, and removing ionic impurities that interfere with IEF. When cells were lysed in "buffers" that support the activity of enzymes such as lysozyme and benzonase, the conductivity of the resulting lysate was an order of magnitude higher than when lysis was induced by chaotropic urea detergent solutions. Following reduction and alkylation, the conductivity of both lysates was lowered by ultrafiltration to the 0.1−0.2 mS/cm range in preparation for IEF. The detergent 3-(4-heptyl)phenyl 3-hydroxypropyl dimethylammonio propanesulfonate (C7BzO), which favors the solubilization of proteins, but which interferes with SDS equilibration and second dimension PAGE, was effectively removed by ultrafiltration and exchanged with CHAPS without measurable loss of protein. Disparate protein patterns of *Rhodopseudomonas palustris* lysates were revealed by two-dimensional gel electrophoresis depending on which reagent was used to induce cell lysis.

Keywords: acrylamide • alkylation • isoelectric focusing • pressure cycling technology • protein solubility • *Rhodopseudomonas palustris* • tributylphosphine • two-dimensional gel electrophoresis • ultrafiltration

Introduction

Sample preparation is crucial to the success of two-dimensional gel electrophoresis (2-DE). Frequently, cells are cultured in media enriched in salts that interfere with IEF. Phosphate is particularly problematic, since it is not effectively removed by acetone precipitation.[1] The use of centrifugal ultrafiltration devices effectively removes these salts, lowers sample conductivity, and facilitates the concentration of proteins for downstream analyses.

For 2-DE, the reduction of protein disulfides must be followed by alkylation to prevent the spurious formation of mixed disulfides during IEF, as they may lead to the formation of unnatural adducts,[2] and to prevent the desulfuration of cysteines that generate labile dehydro alanine residues that are susceptible to cleavage at the peptide bond.[3]

Iodoacetamide (IAA) is relatively inefficient as an alkylating agent. IAA may nonspecifically alkylate lysine and methionine residues in addition to cysteines, and may propagate apocryphal charge trains in two-dimensional gels. Further, IAA is rapidly consumed in the presence of thiourea[4] and its activity is inhibited by SDS and CHAPS.[5] Therefore, IAA is ineffective for alkylation during the SDS equilibration process that precedes second dimension PAGE. By comparison, vinyl monomers such as acrylamide and dimethylacrylamide do not react with methionine and the modification of lysines is a relatively rare event when the alkylation reaction is promptly terminated.[6] Hamdan et al.[7] reported that the reactivity of acrylamide with cysteine sulfhydryls was at least 2 orders of magnitude higher than with lysine NH_2 when the reaction is terminated within a few hours. It is essential to scavenge, or remove completely, the acrylic monomer since increasing numbers of NH_2 groups are alkylated if the reaction is allowed to proceed unchecked.

Reduction can proceed simultaneously with alkylation when nonthiols such as tris(carboxyethyl)phosphine (TCEP) or tributylphosphine (TBP) are substituted for the more commonly used dithiols, which would otherwise scavenge the alkylating agent.[8]

Of possible consequence, Tris or other ionic species with a pK_a that closely matches the pH at which alkylation is favored are frequently used to buffer these reactions, but these ions may interfere with downstream IEF.[9] Therefore, reduction and alkylation performed directly in a centrifugal ultrafiltration device provides a vehicle for both terminating the alkylation reaction and removing ionic species that cause interference in IEF.

Rhodopseudomonas palustris is a gram-negative phototropic bacterium and metabolically versatile microbe. The bacterium can grow in the presence or absence of oxygen. In response to environmental changes, it can engage in alternative metabolic processes for cellular respiration. *R. palustris* can degrade the aromatic compounds comprising lignin, the second most abundant polymer on earth. As such, it is being investigated for its potential in the removal of environmental pollutants.

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Materials and Methods

Materials. *R. palustris* was obtained from Oak Ridge National Laboratories (Oak Ridge, TN). The Barocycler NEP2017 pressure cycling instrument and corresponding processing containers (PULSE Tubes) were from Pressure BioSciences (West Bridgewater, MA). BugBuster Plus Reagent containing recombinant lysozyme and benzonase was obtained from EMD Biosciences (Madison, WI). C7BzO, CHAPS, and TBP were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Ultrafree-0.5 centrifugal ultrafiltration devices were obtained from Millipore Corporation (Billerica, MA). Bradford reagent and AG 501-X8 ion-exchange resin were obtained from BioRad (Hercules, CA). Conductivity was measured with a B-163 conductivity meter from Horiba Instruments (Kyoto, Japan). Immobilized pH gradients (IPGs), second dimension polyacrylamide gels, and ProteomIQ Blue colloidal Coomassie stain were obtained from Proteome Systems (Woburn, MA). The Progenesis Discovery and Editor image analysis software was obtained from Nonlinear Dynamics (Newcastle Upon Tyne, UK).

Cell Lysis Using Pressure Cycling Technology. *R. palustris* cells were pelleted by centrifugation at 12 000 relative centrifugal forces (RCF) for five minutes and suspended at a density of 0.35 g cells/mL in distilled H_2O. For each analysis, 0.57 mL of this suspension (~200 mg cells) was pelleted by centrifugation. One sample was suspended in 1.5 mL of 7 M urea, 2 M thiourea, and 25 mM C7BzO (the C7BzO reagent). The other sample was mixed with 0.75 mL of 2X BugBuster Plus Reagent and the volume was adjusted to 1.5 mL with water. To lyse the cells, 1.5 mL of each sample was placed in a PULSE Tube and subjected to five pressure cycles in the Barocycler NEP2017. Each cycle consisted of 20 s at 35 000 PSI and 20 s at ambient pressure.[10] For each cycle, the pressure reached 35 000 PSI in less than three seconds and returned to ambient in less than 1 s. The resulting lysates were immediately centrifuged at 12 000 RCF to pellet cellular debris. The BugBuster reagent yielded approximately four times more insoluble material than the C7BzO reagent.

Reduction and Alkylation. A 0.1 mL aliquot of each supernatant was mixed with 0.4 mL of 7 M urea, 2 M thiourea, and 65 mM CHAPS (the CHAPS reagent) in an Ultrafree 0.5 mL centrifugal ultrafiltration device (Figure 1) with 10 kDa MW cutoff. Following the addition of 40 mM Tris, 5 mM TBP, and 10 mM acrylamide, the devices were incubated 2 h with slow continuous tube inversions. No leakage of sample into the filtration tube was observed.

Ultrafiltration. To terminate the alkylation reaction, the Ultrafree devices were centrifuged at 10 000 RCF for 10 min or until a retentate volume of approximately 0.1 mL was obtained. Then 0.4 mL of CHAPS reagent that was ion-exchanged with AG 501-X8 resin was added and centrifugation was continued until a retentate volume of approximately 0.1 mL was again obtained. This process was repeated to ensure the complete removal of Tris, TBP, and acrylamide monomer.

Each retentate was transferred to a tared 1.5 mL polypropylene tube, positioned on an analytical balance with a Styrofoam block. The membrane of each Ultrafree device was rinsed with 0.1 mL of CHAPS reagent, and this rinse was combined with its respective retentate. Sample volumes were adjusted to 0.4 mL by adding CHAPS reagent to obtain the appropriate weight (d = 1.12 g/L, 0.4 mL = 0.448 g). For normalizing multiple samples, volumes adjusted by weight had a 1.1% coefficient of variation (n = 10), comparable to the accuracy of a standard pipet.

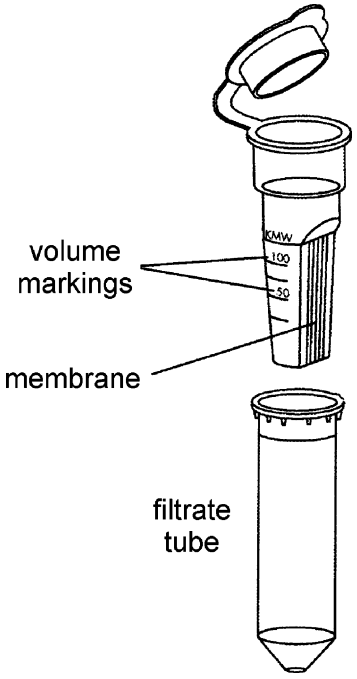

Figure 1. Diagram of the Ultrafree 0.5 mL centrifugal filtration device. The maximum volume that can be applied is 0.5 mL (although larger volumes can be processed by multiple applications). The minimum retention volume is 0.02 mL.

IEF and 2DGE. Dried IPG strips (110 × 3 × 0.3 mm) were hydrated with 0.2 mL of each sample for 4 h prior to IEF for 100 kVh as described.[11] IPGs were equilibrated (2 × 10 min in Tris-acetate pH 7.0 containing 100 mM SDS, 3 M urea, and 0.01% phenol red) prior to running the second dimension PAGE on 6−15% polyacrylamide gradient gels (140 × 100 × 1 mm). Gels were 112 mM Tris-acetate pH 7.0 and contained no SDS. Electrolyte was 50 mM Tris, 50 mM Tricine, and 35 mM SDS pH 8.3. Following electrophoresis, the gels were stained with ProteomIQ Blue colloidal Coomassie stain.[12]

Results and Discussion

BugBuster and C7BzO reagents produced lysates of similar protein concentration, 3.5 and 4.1 mg/mL respectively, as determined by a Bradford assay. The pronounced purple coloration of the BugBuster lysate was indicative of the release of intracellular pigment, presumably bacteriochlorophyll. However, this pigment was largely insoluble and was removed by centrifugation. Residual pigment, not pelleted by centrifugation, accumulated on the membrane of the centrifugal ultrafiltration device resulting in longer filtration times for the BugBuster reagent. It is proposed that the general lack of coloration observed in the C7BzO lysate might be due to the release of magnesium from the porphyrin and its removal by ultrafiltration, or due to an overall increase in the solubility of these proteins. No DNA interference was observed in two-dimensional gels of either lysate, whether benzonase was included.

A similar number of proteins were detected in each lysate, but as Figure 2 clearly illustrates, very different protein profiles were observed in two-dimensional gels. It is hypothesized that the inclusion of lysozyme in the BugBuster reagent may promote lysis of more cells, whereas the C7BzO reagent may have lysed fewer cells but solubilized more proteins, particularly membrane proteins. The BugBuster reagent is optimized to

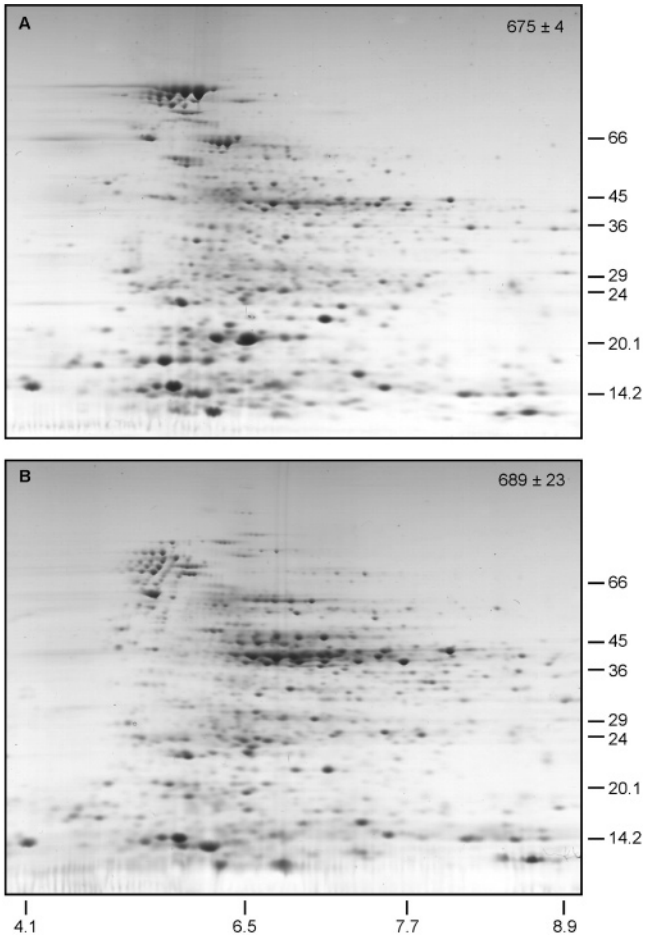

Figure 2. Differential 2-DE patterns of *R. palustris* using (A) C7BzO or (B) the BugBuster Reagent for cell lysis. In duplicate gels of each sample, 675 ± 4 proteins spots were detected in the C7BzO lysate; compared to 689 ± 23 in the BugBuster lysate. For 2-DE, the sample volumes were adjusted to 0.4 mL representing a 1:4 dilution of the original lysate. Estimates of molecular mass (kDa) and p*I* are indicated on the ordinate and abscissa, respectively. IPGs were pH 3−10.

maintain lysozyme activity and was designed for the purification of recombinant proteins, most of which are cytoplasmic. The proprietary BugBuster reagent may not be sufficiently stringent to solubilize membrane proteins, and therefore, it may be biased toward the release of cytoplasmic proteins.

In contrast, Rabilloud et al.[13] demonstrated that C7BzO is highly efficient for solubilizing membrane proteins. This would explain the multiplicity of proteins isolated in the C7BzO lysate that were absent or diminished in the BugBuster lysate. It has been suggested that some proteins initially solubilized in C7BzO, might precipitate when exchanged into a less stringent detergent such as CHAPS. However, no loss of protein was observed experimentally as a result of this exchange. Figure 3 compares two-dimensional gels of *R. palustris* lysates exchanged into 7 M urea, 2 M thiourea containing either 25 mM C7BzO or 65 mM CHAPS, respectively. The integrated spot volume of 573 proteins matched from the C7BzO and CHAPS retentates, when plotted against one another, closely aligned to the theoretical slope of 1.00 (Figure 4, dashed line) suggesting equivalence. While the precise mechanism is not known, it is postulated that C7BzO tenaciously binds hydrophobic protein domains, and is not easily displaced by CHAPS. It is mostly

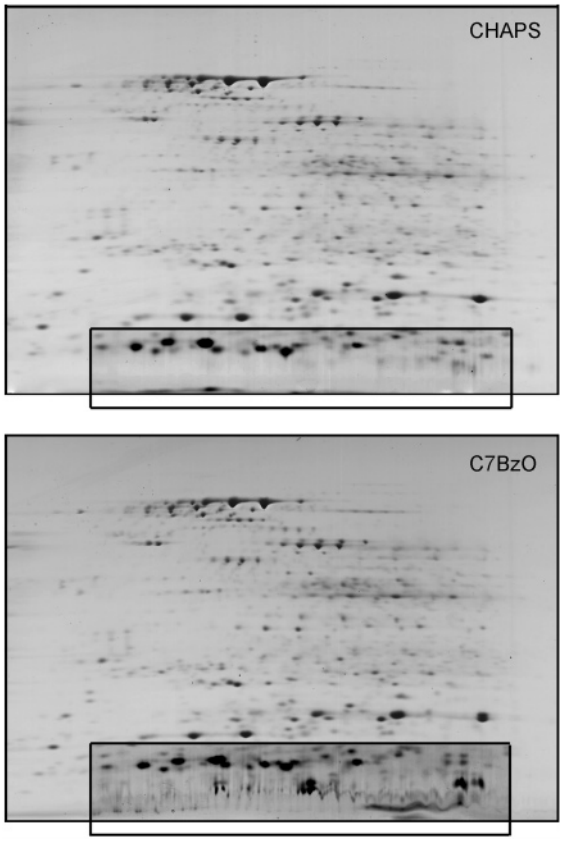

Figure 3. Two-dimensional gels of *R. palustris* lysates exchanged into 7 M urea, 2 M thiourea containing either 65 mM CHAPS (top) or 25 mM C7BzO (bottom). Outlined regions are imaged enhanced to show distortion of low molecular weight proteins in the zone of mixed SDS−C7BzO micelles (bottom gel), which results when extraneous C7BzO is not removed. IPGs were pH 4−7.

the extraneous C7BzO that is removed by ultrafiltration and replaced by CHAPS. It is this extraneous C7BzO that would otherwise remain in the IPG strip, where it would form mixed micelles with SDS during equilibration. The electrophoretic transport of mixed micelles into the second dimension gel can negatively affect the resolution of low MW proteins (Figure 3, bottom gel, outlined region).

Previously, Chernokalskaya et al.[1] showed the deleterious effects of phosphate and other interfering ions carried over from sample preparation on IEF and their efficient removal by ultrafiltration. This was evidenced by the marked decrease in the conductivity of the lysates prior to IEF. The initial conductivity of the BugBuster lysate was 17.4 mS/cm, compared to the C7BzO lysate, which was 1.1 mS/cm. Following ultrafiltration, the conductivity of both lysates was less than 0.2 mS/cm. It is also desirable to remove excess Tris, which was originally added to ensure efficient alkylation, since it forms an ionic boundary that focuses at approximately pH 8.3 at 18 °C. If not removed, Tris may interfere with protein focusing in the alkaline region of the IPG. Tris focuses more rapidly than proteins and produces a localized zone of increased conductivity, the breadth of which directly relates to Tris concentration, corresponding to a precipitous voltage drop in that vicinity. Basic proteins, already near their p*I*, are frequently observed to align at the peripheries of this zone and fail to migrate to the proper position in the pH gradient.[9]

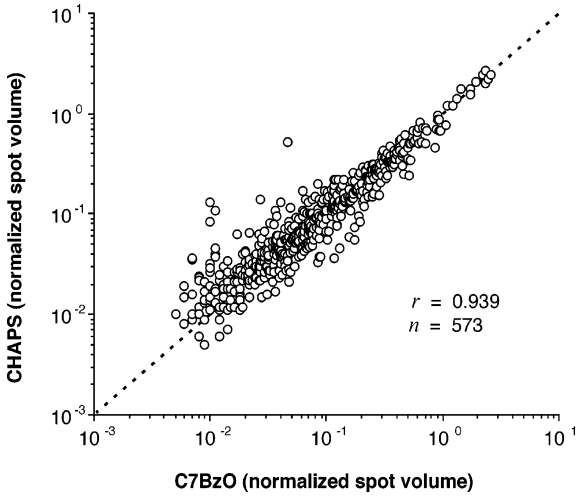

Figure 4. Normalized spot volume of 573 matched spots from duplicate 2D gels of *R. palustris* lysates derived with 25 mM C7BzO, 7 M urea, and 2 M thiourea, following ultrafiltration and exchange into ion-exchanged 7 M urea, 2 M thiourea containing either 25 mM C7BzO (control, abscissa) or 65 mM CHAPS (ordinate). No significant loss of protein based on differences in spot volume was observed as a result of the exchange from C7BzO into CHAPS. A slope (*m*) of 0.88 was observed, close to the expected theoretical slope of 1.00 (dashed line). The outliers above the dashed line represent low molecular weight proteins that were distorted in the zone of mixed SDS−C7BzO micelles, which result when C7BzO is not substituted with CHAPS.

Protein Recoveries from Centrifugal Filtration Devices. The centrifugal devices use Biomax membranes that are designed and thoroughly tested for low protein binding. The protein recovery is reported to be in 85−90% range when tested at low protein concentrations (1 mg/mL) in physiological buffer.[14] However, it is expected that the use of chaotropic reagents will even further reduce protein losses in the devices. Increased yields of both high and low MW proteins from bovine liver lysate concentrated by ultrafiltration has been reported, compared to acetone precipitation, where the selective loss of endoplasmic reticulum membrane proteins of the disulfide isomerase family was observed.[1,15] The depletion of high MW proteins by acetone precipitation of *Fasciola hepatica* was also reported Jefferies et al.[16] Similarly, Yeoman et al.[17] recovered more proteins from *Streptomyces thermovulgaris* by ultrafiltration than by acetone precipitation. Moreover, ultrafiltration has been used as an alternative to dialysis to prepare cerebrospinal fluid from multiple sclerosis patients for 2-DE.[18,19]

Acrylamide Alkylation Reaction. To obtain reliable IEF, it is critical that the acrylamide alkylation reaction is terminated within a few hours, or the specificity of acrylamide alkylation is curtailed. There is an increase in the number of amines that are alkylated over time, and hence, a gradual shift in protein pI toward the electronegative occurs. The reaction may be terminated by removing the vinyl monomer by centrifugal ultrafiltration, as was done in this experiment, or by the addition of a molar excess of dithiothreitol (DTT) which scavenges the residual acrylamide monomer in a redox reaction. However, this approach further increases sample conductivity. Acrylamide monomer can also be removed by precipitating the proteins in organic solvents, such as acetone or methanol:chloroform, but there is a chance that extremely hydrophobic proteins might partition into the organic phase, which would skew the representation of released proteins.[20]

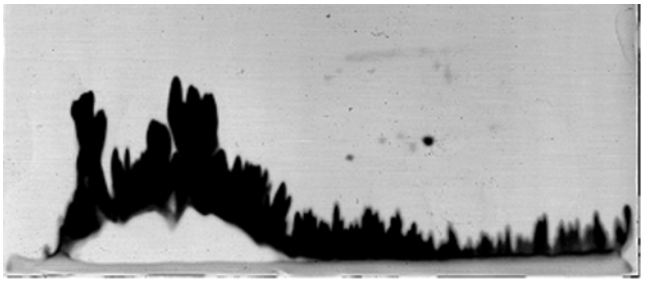

Figure 5. Enlargement of lower region of two-dimensional gel showing the mixed SDS−C7BzO micellar front. The hydrophobic micellar front is stained with ProteomIQ Blue when the gel is not fixed prior to staining. IPG was pH 3−5. It is hypothesized that the apparent increase in mixed micelle formation at the acidic extreme of the IPG (left) is related to a slower rate of SDS impregnation due to repulsion by immobilized negative charges.

Other Redox Reagents. Typically, acrylamide alkylates with only 80% efficiency.[7] Sebastiano et al.[21] showed that 100% alkylation efficiency was possible with 2- or 4-vinylpyridine, but like acrylamide, the reaction must be controlled to prevent the eventual alkylation of lysines. Bai et al.[22] used TCEP and 2-vinylpyridine as a redox couple to avoid issues with TBP toxicity and instability. Within minutes, TBP is almost completely oxidized to Bu_3PO.[23] The vinylpyridine reaction can also be terminated by the addition of a molar excess of DTT, or more simply, by its removal by ultrafiltration.

Detergent Removal by Ultrafiltration. Ultrafiltration of C7BzO lysates and exchange into CHAPS buffer diminishes the formation of mixed detergent micelles that accumulate behind the Kolrausch boundary[24] in second dimension SDS-PAGE and skews the separation of low MW proteins (Figure 5). C7BzO has a critical micelle concentration (CMC) in the micromolar range, and therefore it occurs almost entirely in micellar form in the IPG and it is not effectively removed during the SDS equilibration preceding PAGE. Consequently, some SDS monomer is incorporated into the otherwise charge-balanced C7BzO micelles and the resulting mixed detergent micelle, now charged, is electrophoretically transported into the second dimension gel. This is in antithesis to what occurs in micellar electrokinetic chromatography where uncharged species are separated by virtue of their sequestration into charged SDS micelles. While its mean aggregation number has not been reported, the micellar MW of C7BzO is apparently less than 10 kDa as evidenced by its rapid removal using centrifugal ultrafiltration with a 10 kDa MWCO membrane. Considering that the formula weight of C7BzO is 399.6 Da, a mean aggregation number less than 25 is predicted for C7BzO in 7 M urea, and 2 M thiourea.

The efficiency of detergent removal appears to be related to CMC. Detergents with a high CMC are most rapidly removed by ultrafiltration, even when the aggregation number and micellar MW exceed the filter pore size, while detergents with a low CMC are more difficult to remove. For example, SDS is effectively removed in a 10 kDa ultrafiltration device, despite the fact that its micellar MW is approximately 18−20 kDa.[25] This is because the CMC of SDS is on the order of 16 mM in 6 M urea and approximately 22% of the total detergent mass is monomeric at 70 mM SDS concentration. As monomers freely pass through the membrane and into the filtrate, thermodynamics drives the disaggregation of micelles in the retentate maintaining a constant monomer concentration equal to the CMC. Meanwhile, as SDS monomer accumulates in the filtrate,

it soon reaches a concentration that exceeds the CMC, and micelles are again formed. Further, SDS removal is expedited when the retentate is supplemented with 10% acetonitrile.[13] This is because CMC increases as the molar fraction of water is decreased. In chaotropic solutions, for example, the CMC of SDS increases 16% and 104% at 2 M and 6 M urea concentrations, respectively.[26]

Other detergents are not as efficiently exchanged by ultrafiltration. The amidosulfobetaine ASB-14 has a high micellar MW and a low CMC and is not removed by ultrafiltration using a 10 kDa membrane. The detergent is almost entirely in micellar form, and consequently, ASB-14 accumulates in the retentate. (Based on the CMCs of similar ASBs synthesized by Rabilloud et al.,[27] the CMC of ASB-14 may be in the micromolar range.) Experimentally, nearly 80% of the ASB-14 remains in the IPG following two 10 minute equilibrations in SDS buffer. As a result, there is a propensity toward the formation of mixed SDS-ASB micelles, which are transferred to the second dimension gel. While ASB-14 and C7BzO are highly recommended for 2-DE,[13,28,29] these literature have largely ignored the potential interference of these detergents in second dimension PAGE.

Conversely, the high micellar MW of ASB-14 could be exploited for the isolation of peptides by ultrafiltration, since the retention of most of the detergent in the retentate would yield a peptide fraction (the filtrate) that is relatively free of this detergent. On the basis of its formula weight of 434.7 Da, and on its inability to pass through a 10 kDa membrane, an aggregation number greater than 23 is predicted for ASB-14. In contrast, CHAPS has a CMC of 6−10 mM and aggregation number of 10, and correspondingly, a small micellar MW (6150 Da). At 65 mM CHAPS concentration, there is approximately 6 mM molar concentration of micelles, or nearly a 1:1 ratio of monomers to micelles (monomers may slightly outnumber micelles).

Conclusions

Reduction and alkylation performed in a centrifugal ultrafiltration device provides an effective means of terminating the alkylation reaction while removing interfering substances and lowering sample conductivity prior to IEF. When nonthiol reagents such as TBP and TCEP are used, simultaneous reduction and alkylation is possible. However, the alkylation reaction must be terminated to prevent artifactual alkylation of lysine residues. This is frequently done by adding a molar excess of DTT, but with the compromise of increasing sample conductivity. When performed entirely in a centrifugal ultrafiltration device, sample manipulations are limited and the processing of numerous samples is likely to be more reproducible. Small volumes are easily processed, while minimizing sample losses and larger samples of low protein concentration can be concentrated in the same device.

Abbreviations. ASB, amidosulfobetaine; C7BzO, 3-(4-heptyl) phenyl 3-hydroxypropyl dimethylammonio propane sulfonate; IAA, iodoacetamide; TBP, tributylphosphine; TCEP, tris(carboxyethyl) phosphine.

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