

# Effective Protein Separation by Coupling Hydrophobic Interaction and Reverse Phase Chromatography for Top-down Proteomics

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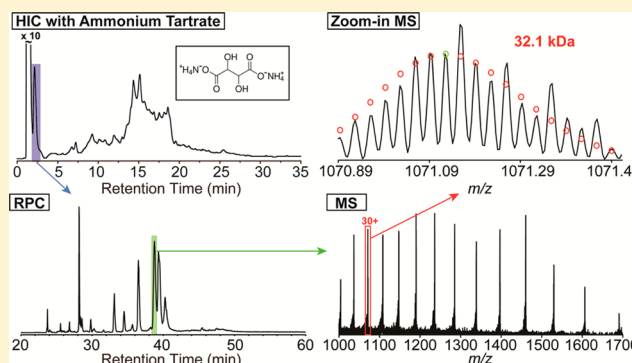
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## S Supporting Information

**ABSTRACT:** One of the challenges in proteomics is the proteome's complexity, which necessitates the fractionation of proteins prior to the mass spectrometry (MS) analysis. Despite recent advances in top-down proteomics, separation of intact proteins remains challenging. Hydrophobic interaction chromatography (HIC) appears to be a promising method that provides high-resolution separation of intact proteins, but unfortunately the salts conventionally used for HIC are incompatible with MS. In this study, we have identified ammonium tartrate as a MS-compatible salt for HIC with comparable separation performance as the conventionally used ammonium sulfate. Furthermore, we found that the selectivity obtained with ammonium tartrate in the HIC mobile phases is orthogonal to that of reverse phase chromatography (RPC). By coupling HIC and RPC as a novel two-dimensional chromatographic method, we have achieved effective high-resolution intact protein separation as demonstrated with standard protein mixtures and a complex cell lysate. Subsequently, the separated intact proteins were identified by high-resolution top-down MS. For the first time, these results have shown the high potential of HIC as a high-resolution protein separation method for top-down proteomics.



Top-down proteomics promises a full description of the proteome including the identification, characterization, and quantification of various proteoforms arising from genetic variations, alternatively spliced RNA transcripts, and post-translational modifications.<sup>1–6</sup> Thus, it has high potential for elucidation of cellular pathways, disease mechanisms, and biomarker discovery, as showcased by recent studies using single proteins and simple protein mixtures.<sup>3,7–12</sup> However, challenges remain to enable top-down proteomics for routine proteome-wide investigation to the same extent as bottom-up proteomics. One of the challenges is the proteome's complexity, which necessitates the fractionation of intact proteins prior to the mass spectrometry (MS) analysis.<sup>12</sup> While effective methods exist for fractionation of small peptides in the bottom-up approach, separation of intact proteins remains challenging despite recent advances in top-down proteomics studies.<sup>1,13–19</sup> Most protein separation/purification methods employ salts and/or detergents that are incompatible with MS.<sup>12</sup> Hence, new chromatographic methods for effective high-resolution protein separations that are compatible with top-down MS are needed.

Hydrophobic interaction chromatography (HIC)<sup>20–23</sup> appears to be the chromatography mode that provides high-resolution separation of the greatest number of intact protein samples.<sup>24</sup> HIC is a nondenaturing mode that separates proteins based on

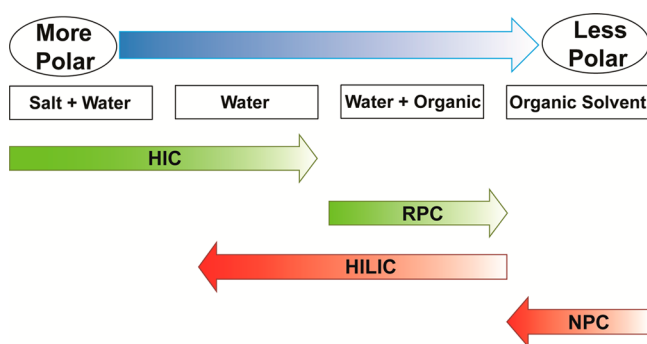
the differences in hydrophobicity on the surface of their tertiary structures.<sup>21,22</sup> Proteins are eluted in the order of increasing surface hydrophobicity by decreasing the salt concentration of the mobile phase. The salt concentration in HIC can be conveniently manipulated to ensure retention of hydrophilic proteins and elution of hydrophobic ones. Scheme 1 compares HIC with the other modes of chromatography that are sensitive to differences in polarity: reverse phase chromatography (RPC), hydrophilic interaction chromatography (HILIC), and normal phase chromatography (NPC). A unique advantage of HIC is that it is a very mild method for high-resolution protein separation in a nondenaturing mode and preserves proteins' tertiary structure and biological activity.<sup>25,26</sup> Moreover, the selectivity of HIC is complementary to those of other chromatographic modes such as ion exchange (IEC), size exclusion (SEC), and affinity chromatography.<sup>25</sup> Unfortunately, proteins are best retained in HIC with high concentrations of nonvolatile salts high in the Hofmeister (lyotropic) series (which classifies ions in order of their ability to salt-out or salt-in proteins), such as ammonium

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**Scheme 1. Comparison of Chromatographic Methods for Separations Based on Differences in Polarity<sup>a</sup>**



<sup>a</sup>Green and red arrowheads indicate the direction of gradient polarity during elution. HIC, hydrophobic interaction chromatography; RPC, reverse phase chromatography; HILIC, hydrophilic interaction chromatography; NPC, normal phase chromatography.

sulfate and sodium sulfate,<sup>27</sup> rendering HIC incompatible with direct MS analysis. On the other hand, salts more compatible with and commonly employed for MS, such as ammonium acetate, are much less able to order the structure of water in their solutions,<sup>28</sup> so the retention of proteins with such salts is weak. Therefore, if we could identify a salt that can confer good retention of proteins in HIC yet does not interfere with MS analysis, we would enable the effective application of HIC to top-down proteomics.

In this study, we have identified ammonium tartrate  $[(\text{NH}_4)_2\text{C}_4\text{H}_4\text{O}_6]$  as a MS-compatible salt that affords high-resolution protein separations in HIC comparable to those obtained with the commonly used ammonium sulfate. Furthermore, we found that HIC with ammonium tartrate in the mobile phase is orthogonal to RPC, despite the fact that retention via both methods is based on hydrophobicity. RPC is the most popular MS-friendly separation method, permitting direct online MS analysis after RPC separation. So it is commonly used as the last dimension before MS, often coupled with IEC,<sup>29,30</sup> SEC,<sup>16</sup> and more recently HILIC.<sup>31</sup> Here, we have coupled HIC with RPC as a novel two-dimensional chromatographic method and achieved effective high-resolution intact protein separation, as demonstrated with standard protein mixtures and a complex cell lysate. Subsequently, the separated intact proteins were identified by top-down MS. For the first time, with the assistance of ammonium tartrate as the mobile phase salt, we have overcome the challenge of MS-compatibility due to the high concentration of nonvolatile salts in HIC and demonstrated the high potential of HIC for top-down proteomics.

## MATERIALS AND METHODS

**Chemicals and Reagents.** All reagents were purchased from Sigma-Aldrich Inc. (St. Louis, MO) unless noted otherwise. HPLC grade water and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ), and BugBuster master mix cell lysis buffer was purchased from EMD Millipore (Novagen, Billerica, MA).

**Sample Preparation.** Standard protein samples (Apr, aprotinin from bovine lung; Cyt, cytochrome C from equine heart; RiA, ribonuclease A from bovine pancreas; Myo, myoglobin from equine heart; RiB, ribonuclease B from bovine pancreas; ChA,  $\alpha$ -chymotrypsinogen A from bovine pancreas; Chy,  $\alpha$ -chymotrypsin from bovine pancreas; Oval, ovalbumin

from chicken egg white; BSA, albumin from bovine serum; Con, conalbumin from chicken egg white) were used without further purification. For HIC, all standard protein samples were first prepared in 10 mg/mL with HPLC-grade water and subsequently diluted to 0.1–1.5  $\mu\text{g}/\mu\text{L}$ . For RPC-MS, the optimized concentration of each standard protein was 0.1–0.2  $\mu\text{g}/\mu\text{L}$  in the four-protein mixture (BSA, Myo, Oval, and ChA) and six-protein mixture (Cyt, Myo, Con, Oval, RiA and RiB), and 1  $\mu\text{L}$  of sample was injected on the RP column for separation.

*Escherichia coli* (*E. coli*) cells from the BL21 strain grown in-house were lysed in BugBuster master mix cell lysis buffer wherein 1 mL of buffer was added to 200 mg of *E. coli* cells followed by the addition of protease and phosphatase inhibitor cocktail and PMSF (100 mM). The lysed cells were resuspended, vortexed, and then shaken for an hour at 4 °C. The resulting lysate was centrifuged at 4 °C for 30 min at 13.2 rpm, and the supernatant solution comprising the complex mixture of *E. coli* cell lysate soluble proteins was utilized for further chromatographic separations. The pellet was discarded.

**Hydrophobic Interaction Chromatography (HIC).** HIC was performed on a Shimadzu HPLC system (Shimadzu Scientific Instruments Inc., Columbia, MD) equipped with a PolyPROPYL A column (100 mm  $\times$  4.6 mm i.d., 3  $\mu\text{m}$ , 1500 Å; PolyLC Inc., Columbia, MD). Ammonium acetate, ammonium sulfate, and ammonium tartrate were utilized as salts for HIC separation of standard protein samples; only ammonium tartrate was used for HIC of *E. coli* cell lysate samples. Mobile phase A (MPA) and mobile phase B (MPB) contain 1.8 M and 20 mM salt, respectively. Both solutions were adjusted with 10% ammonium hydroxide ( $\text{NH}_4\text{OH}$ ) solution to pH 7.0. Typically, a 30 min linear gradient (from 100% MPA to 100% MPB) was utilized to elute proteins followed by MPB isocratically for 5 min to ensure elution, at a flow rate of 1 mL/min. All samples were diluted with MPA at the volume ratio 1:1 to avoid injection viscosity differences. For standard protein samples, the gradient profile was slightly optimized to achieve a better separation: two isocratic regions from 12 to 14.5 min (at the proportion of 48.3% MPB) and 15 to 19 min (63.3% MPB) were interjected. The sample injection volume was 50  $\mu\text{L}$ . Baseline subtraction was performed for all HIC chromatograms. Other chromatographic conditions are given in the figure legends.

**Reverse Phase Chromatography (RPC).** RPC was performed on a Thermo EASY nano-LC 1000 system (Thermo Fisher Scientific) equipped with a PicoFrit PLRP-S column (100 mm  $\times$  100  $\mu\text{m}$  i.d., 5  $\mu\text{m}$ , 1000 Å; New Objective, Inc., Woburn, MA). The following buffers were used for RPC: buffer A, water with 0.25% formic acid; buffer B, acetonitrile with 0.25% formic acid. The nano-LC was operated at a flow rate of 500 nL/min, and 1  $\mu\text{L}$  of sample was injected with an autosampler after equilibration of the capillary column. For the separation of both standard proteins and *E. coli* cell lysate proteins, an 80 min optimized RPC gradient was used consisting of the following concentrations of buffer B: 5% for 15 min, 25% at 25 min, 60% at 70 min, 95% at 75 min, and then back to 5% at 80 min. Prior to injection on the RPC column, a brief desalting procedure (three times with 10 kDa ultracentrifugal filters) was performed on both the *E. coli* cell lysate fractions after HIC with ammonium tartrate salt gradient and standard proteins prepared in 1.8 M ammonium tartrate buffer to remove a substantial amount of salt.

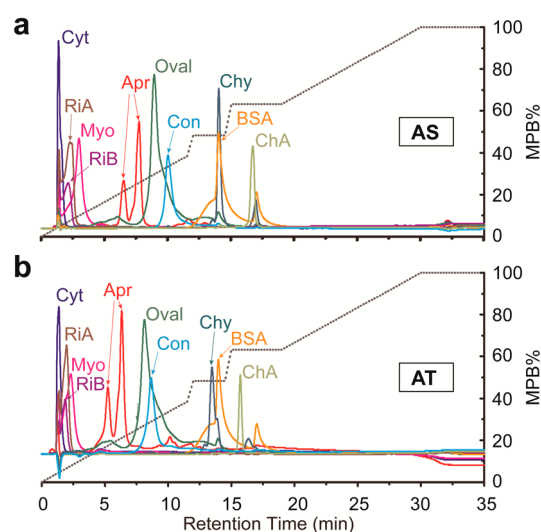
**Top-down MS Analysis.** For online nano-LC/MS/MS experiments, samples were electrosprayed<sup>32</sup> with a “nanoflex” ionization source into a Q Exactive benchtop Orbitrap mass

spectrometer (ThermoFisher Scientific, Bremen, Germany).<sup>33,34</sup> Heated metal capillary temperature and s-lens voltage were experimentally optimized to 300 °C and 50 V, respectively, for more energetic front-end source conditions for improved sensitivity, ion transmission, and reduced adduction.<sup>35–39</sup> Both LC/MS and LC/MS/MS data were collected with five micro scans at a high mass resolving power of 140 000 (theoretical maximum resolving power setting on QE:  $m/\Delta m_{50\%} = 140\,000$  at  $m/z$  200, in which  $\Delta m_{50\%}$  denotes mass spectral peak full width at half-maximum peak height) and 70 000, respectively. In top two data-dependent MS/MS scans, the intact protein ions are injected into the collision cell for higher-energy collision dissociation (HCD)<sup>40,41</sup> at a previously optimized setting of 23 V, and all ions moved back into the C-trap and then into the Orbitrap for analysis. Here, intact protein ions with greater than 8 positive charges were quadrupole-isolated for HCD, and other ions with fewer charges were discounted. Data were collected with Xcalibur 2.2 software (Thermo Fisher Scientific, Bremen, Germany), and the total RPC-MS data acquisition period was 80 min per sample.

**Protein Identification.** Raw top two data-dependent MS data collected by Xcalibur were first processed with MS-Deconv, a combinatorial algorithm<sup>42</sup> for reliable isotopic distribution deconvolution and charge state assignment<sup>43</sup> for all the observed ions to generate MSAlign files containing monoisotopic mass, intensity, and charge. These MSAlign files were then subsequently searched with the *E. coli* BL21 database generated from NCBI (accession: PRJEA161949, containing 4192 protein sequences) with the alignment-based MS-Align+ algorithm for protein identifications based on protein-spectrum matches.<sup>44</sup> Here, 10 ppm fragment mass tolerance searches were conducted for the assignment of *b* and *y* ions. Protein identification results with statistically significant lower *P* and *E* values (<0.00005) and a satisfactorily higher fragment number (at least 10 fragments assigned) were manually validated.

## RESULTS AND DISCUSSION

**Identification of Ammonium Tartrate as a MS-Compatible Salt for HIC.** To identify a salt that combines MS compatibility downstream with high-resolution separation, a set of standard proteins was used initially. First, we used the commonly used HIC salt, ammonium sulfate, to optimize separation conditions and demonstrate the excellent retention and selectivity for proteins in HIC.<sup>27</sup> An overlay of individual injections of the 10 standard proteins reveals satisfactory peak shapes for a majority of the proteins (Figure 1a). Inspired by the excellent performance of ammonium tartrate as a MS-compatible additive for top-down MS of large proteins,<sup>45</sup> and considering the fact that tartrate is high in the Hofmeister series, we hypothesized that ammonium tartrate could be adapted for use in HIC as a potential MS-compatible salt. Accordingly, we assessed its HIC performance via individual injections of the same set of 10 standard proteins with ammonium tartrate as the salt (Figure 1b). The results were comparable to those obtained with ammonium sulfate (Figure 1a). The similarities of sulfate and tartrate can be partially explained by the Hofmeister (lyotropic) series, which categorizes ions in order of their ability to precipitate (salt-out) or solubilize (salt-in) proteins.<sup>27,28,46,47</sup> Sulfate and tartrate are similarly positioned in the Hofmeister series, and both are doubly charged anions effective for salting-out proteins. They can interact very unfavorably with hydrocarbon groups on proteins and therefore stabilize folded proteins relative to the unfolded form in which more hydrocarbon groups are



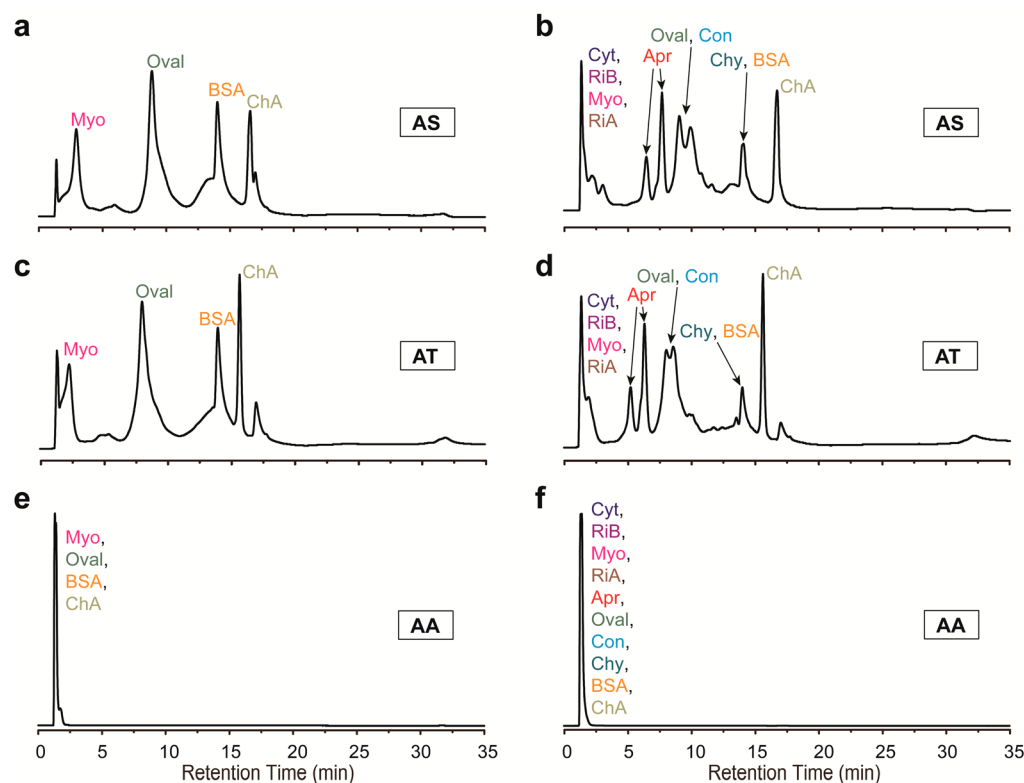
**Figure 1.** Overlay of HIC chromatograms of individual standard proteins in different mobile phases containing (a) ammonium sulfate (AS) and (b) ammonium tartrate (AT). Conditions: PolyPROPYL A column, 100 mm  $\times$  4.6 mm i.d., 3  $\mu$ m, 1500 Å; MPA, 1.8 M salt aqueous solution at pH 7.0; MPB, 20 mM salt aqueous solution at pH 7.0; column temperature, 25 °C; UV detection, 280 nm; flow rate, 1 mL/min; gradient, 30 min from 100% MPA to 100% MPB. The gray dash line refers to the MPB percent in the gradient profile. Apr, aprotinin; Cyt, cytochrome C; Myo, myoglobin; Oval, ovalbumin; BSA, bovine serum albumin; RiB, ribonuclease B; RiA, ribonuclease A; Chy,  $\alpha$ -chymotrypsin; ChA,  $\alpha$ -chymotrypsinogen A; Con, conalbumin.

exposed.<sup>27,28,46–48</sup> For the purpose of serving as effective HIC salts, other properties, such as the acid–base dissociation constant (thus the pH values of the mobile phase) and the solubility, are also important factors.

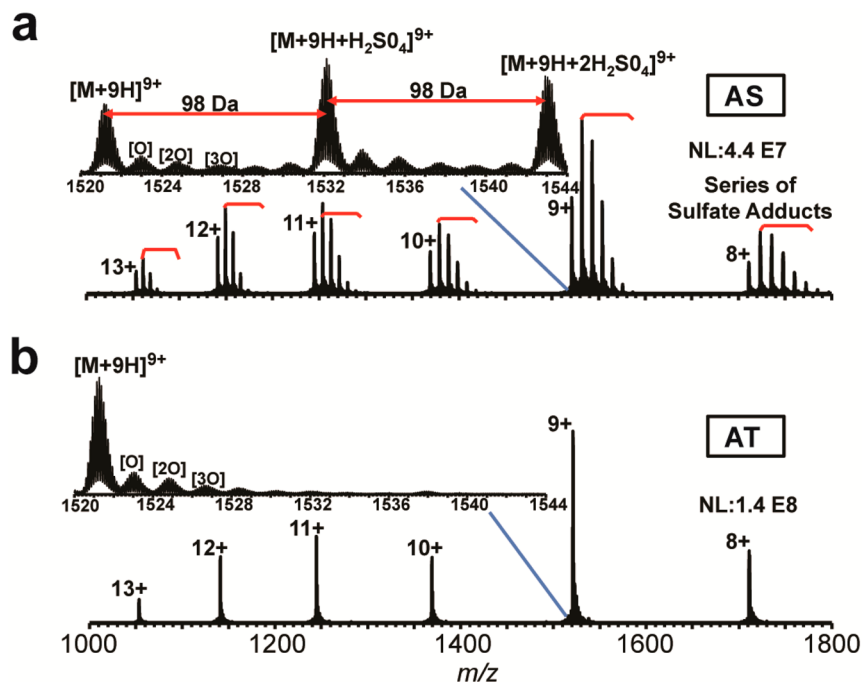
These two salts were compared further with a mixture of four of the protein standards (“4-mix”; BSA, Myo, Oval, and ChA) and 10 of the standards (“10-mix”; all ten of the protein standards featured in Figure 1). The concentration of each protein was the same as the concentration with individual injections. Standard proteins in the 4-mix and 10-mix samples (Figure 2) were identified according to retention time in reference to the individual protein injections in Figure 1. Similar separation profiles were obtained for HIC using ammonium sulfate (Figure 2a,b) as compared to ammonium tartrate (Figure 2c,d), for 4-mix and 10-mix, respectively. Nearly all proteins were baseline separated for 4-mix (Figure 2a,c), whereas only a portion of proteins was separated in 10-mix (Figure 2b,d). The less hydrophobic proteins (Cyt, Myo, RiA, and RiB) tend to coelute under the conditions used here.

We further compared the performance of ammonium tartrate with ammonium acetate, a commonly used MS-friendly salt, for HIC. A clear contrast was found in chromatograms of both 4-mix (Figure 2c,e) and 10-mix (Figure 2d,f). Ammonium acetate failed to retain any proteins resulting in an extremely high intensity peak due to the coelution of the entire protein mixture at the beginning of the chromatography runs. This can be explained by acetate ion’s low position in the Hofmeister series,<sup>27</sup> denoting relatively poor water-structuring power. Thus, despite its compatibility with MS, ammonium acetate is not suitable for use as a salt with a conventional HIC stationary phase, as has been noted before.<sup>49</sup>

Next, we investigated the MS-compatibility of ammonium tartrate in the online LC/MS mode compared with the



**Figure 2.** HIC separations of standard protein mixtures with ammonium sulfate (a,b), ammonium tartrate (c,d), and ammonium acetate (e,f). HIC UV-chromatograms of 4-mix sample is shown in the left column (a, c, and e) and 10-mix sample is depicted in the right column (b, d, and f). Conditions: same as shown in Figure 1.

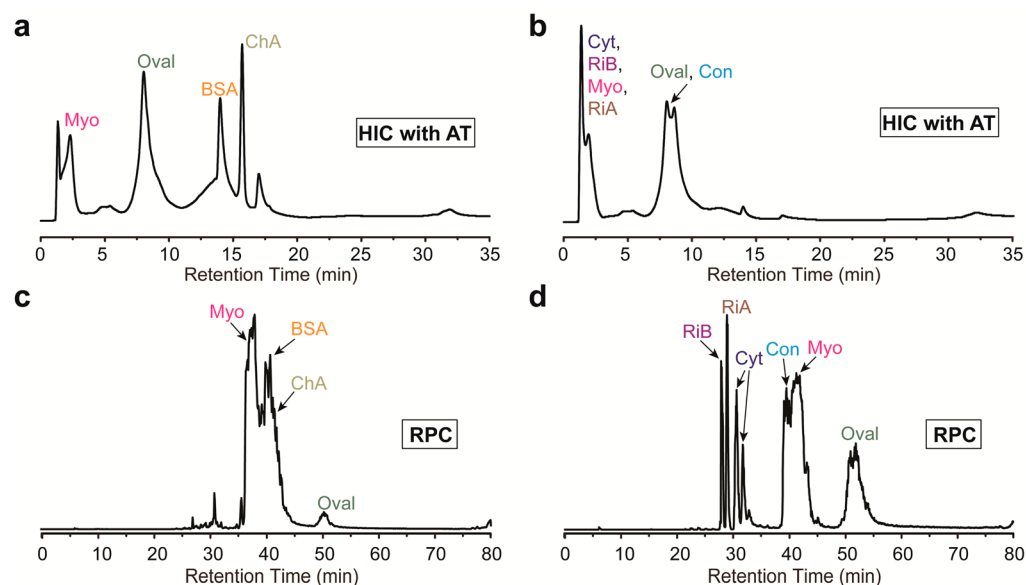


**Figure 3.** Evaluation of MS-compatibility of HIC salts. Representative mass spectra of ribonuclease A in HIC buffers: (a) 1.8 M AS, (b) 1.8 M AT, desalted by ultracentrifugation device and RPC. NL, normalized level.

conventional HIC salt, ammonium sulfate. Standard proteins were prepared in the mobile phase containing 1.8 M (the highest concentration used in HIC fractions) ammonium tartrate or ammonium sulfate and then desalted briefly by an ultracentrifugation step with a 10-kDa filter (3 cycles) prior to

RPC/MS analysis. Significant sulfate adducts of 98 Da and concomitant reduced S/N were observed for standard proteins prepared in ammonium sulfate (representative spectra shown in Figure 3a and Figure S-1a). In contrast, high quality spectra with no adduction and improved S/N were obtained for the

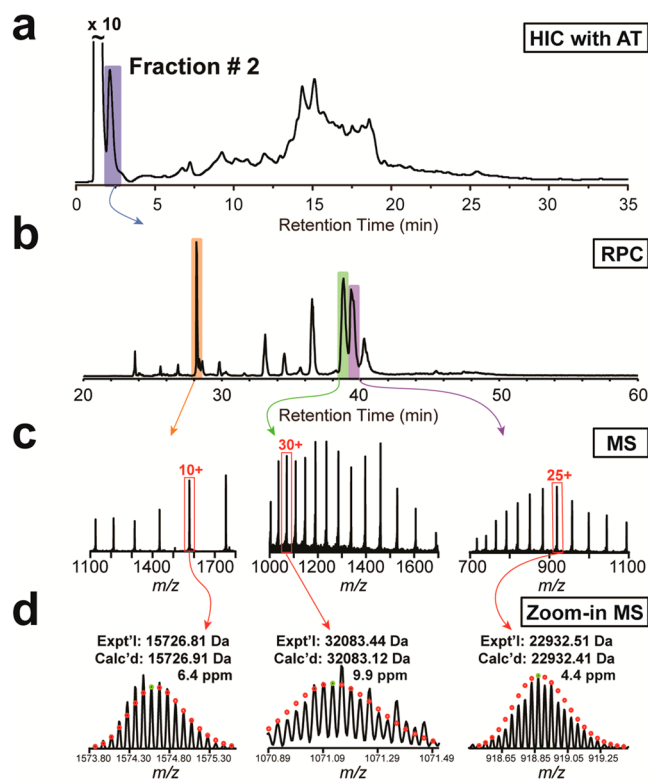




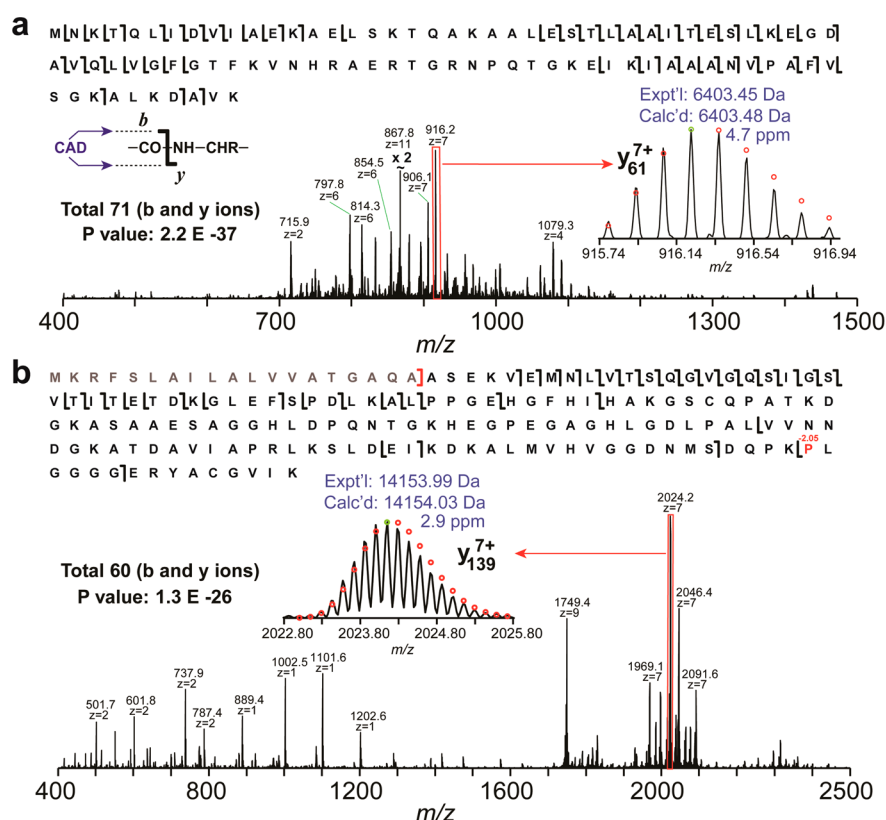
**Figure 4.** Separation of standard protein mixtures by HIC using ammonium tartrate as salt (a and b) and RPC (c and d), suggesting the orthogonality between HIC and RPC. (a) and (c), 4-mix (Myo, Oval, BSA, ChA). (b and d) 6-mix (Cyt, RiB, Myo, RiA, Oval, Con). HIC conditions, same as shown in Figure 1. RPC conditions, PicoFrit column (New Objective) PLRP-S, 100  $\mu\text{m}$  i.d.  $\times$  100 mm, 5  $\mu\text{m}$ , 1000 Å; MPA, water with 0.25% formic acid; MPB, acetonitrile with 0.25% formic acid; column temperature, 25  $^{\circ}\text{C}$ ; flow rate, 500 nL/min. Gradient: Initial 15 min isocratic segment of 5% MPB, followed by linearly increasing MPB to 25% in 10 min, then to 60% linearly in 45 min, to 95% in the next 5 min, and finally decreased to 5% in 5 min. Total: 80 min.

same standard proteins from the solution of ammonium tartrate (Figure 3b and Figure S-1b). Ammonium tartrate (0.5–25 mM) has been previously utilized as an additive in direct infusion mode for improved top-down dissociation of large intact proteins ( $\sim$ 200 kDa) by McLafferty and co-workers<sup>45</sup> as well as for reduction of sodium ion adduction on protein ions in native MS by Williams and co-workers.<sup>38</sup> We have confirmed the MS-compatibility of ammonium tartrate via direct infusion of standard proteins in various concentration of ammonium tartrate (up to 50 mM), and the resulting ESI/FTMS spectra yield clean charge state distribution without any adduction (data not shown). These results further underscore the MS-compatibility of ammonium tartrate.

**HIC and RPC Orthogonality.** Since it is likely that no single dimension of LC suffices to resolve complex protein mixtures, we sought to employ a second dimension of LC after the first dimension separation by HIC. RPC is commonly used as the second (or last) dimension of chromatographic separation prior to MS. Although both HIC and RPC separate proteins based on difference in hydrophobicity, selectivity in RPC differs from HIC because the conditions of RPC denature proteins, giving the column access to a different set of hydrophobic residues than is available in the nondenaturing mode of HIC. This provides the possibility of orthogonality between HIC and RPC. These two modes also use different mobile phases with different polarity (as illustrated in Scheme 1). Thus, we evaluated the possibility of coupling HIC with RPC for two-dimensional chromatographic separation using standard protein mixtures. A mixture of four proteins, specifically Myo, Oval, BSA, and ChA, was nicely separated by HIC with ammonium tartrate as the salt (Figure 4a), whereas RPC was unable to separate the same protein mixture (Figure 4c), especially BSA and ChA. On the other hand, another protein mixture, containing Cyt, Myo, RiA, RiB, Oval, and Con could not be resolved in HIC (Figure 4b) but was successfully separated by RPC (Figure 4d). These results confirm the complementary selectivity of the two modes.



**Figure 5.** HIC-RPC separation and MS analysis of *E. coli* cell lysate samples. Representative RPC and MS data from one HIC fraction are shown. (a) UV-chromatogram obtained for *E. coli* cell lysate by HIC separation with ammonium tartrate as the gradient salt HIC conditions, same as shown in Figure 1, except the gradient profile simplified to a 30 min linear gradient from 100% MPA to 100% MPB. (b) RPC-MS TIC for HIC fraction 2 after ultracentrifugal desalting. RPC conditions, same as shown in Figure 3. (c and d) Representative mass spectra for three *E. coli* proteins observed in the HIC-RPC MS platform with charge state distributions (no tartrate adduction) and unit mass isotopic resolution on a chromatography time scale on Q Exactive.



**Figure 6.** Online RPC/MS/MS protein identifications by HCD for HIC fraction 2. Representative MS/MS spectra and sequence maps of identified proteins with *b/y* ion cleavages and *P* values for protein identification. The insets highlight the isotopic resolution for representative fragments at *m/z* 916 and 2024, respectively.

**HIC/RPC/Top-down MS for Identification for *E. coli* Proteins.** This HIC-RPC-MS sequence was next applied to a more complex mixture of an *E. coli* cell lysate. Proteins were eluted with a decreasing ammonium tartrate salt gradient, and fractions were collected every minute (Figure 5a). To demonstrate proof-of-principle for this combination, we focused on HIC fraction 2 for the subsequent RPC/MS analysis since the early HIC fractions contain a very high concentration of ammonium tartrate (~1.7 M), which appears to be more challenging to handle than the later fractions. Fraction 1 was not chosen because it contained highly abundant peptides that are not retained by HIC. The RPC total ion current (TIC) chromatogram obtained for HIC fraction 2 clearly shows baseline separation of a number of proteins, demonstrating the power of the two-dimensional separation (Figure 5b). The online RPC/MS analysis shows high-resolution MS measurement of intact proteins without adduct formation. Representative data for three proteins, 15.7 kDa, 22.9 kDa, and 32.1 kDa, are shown in Figure 5c,d. At the resolving power utilized, isotopic clusters for intact proteins up to ~32 kDa were clearly evident on the Q Exactive MS. The experimental and calculated isotopic distributions agree well for observed intact proteins (Figure 5d). The MASH-suite software<sup>50</sup> was used to generate the calculated isotopic distribution based on the observed molecular weights and averaging model.<sup>43</sup> Generally, proteins below ~20 kDa are isotopically resolved in a single LC-MS scan at maximum resolving power setting (140 K at *m/z* 200) on the Q Exactive, but one needs to signal-average more scans to clearly define the isotopic distribution of larger proteins and improve S/N. Here, 5, 10, and 15 scans are signal-averaged in the chromatographic time scale to improve S/N and define a

Gaussian isotopic distribution as shown for 15.7 kDa, 22.9 kDa, and 32.1 kDa intact proteins, respectively (Figure 5d). The lack of isotopic resolution of large molecular weight (MW) species beyond 32 kDa might reflect the upper MW limit that can be resolved online by a Q Exactive because of the exponential decay in S/N vs increasing MW<sup>51</sup> and/or the rapid decay in the time-domain signal due to overall higher kinetic energies of ions in the Orbitrap.<sup>52</sup>

Since accurate MW measurement of intact protein mass by MS is not conclusive for unambiguous protein identification, MS/MS is required to obtain sequence information so to identify the proteins from the database. Here, we have employed top-2 data-dependent HCD in the chromatographic time-scale for each HIC fraction separated by RPC as the second dimension for protein identifications. Similarly to collisionally activated dissociation (CAD), HCD mostly produces *b* and *y* ions by the cleavage of the peptide backbone amide bond.<sup>40,41</sup> Figure 6 illustrates a single scan HCD mass spectrum and also *b/y* ions, cleavages observed by HCD for two representative *E. coli* proteins identified by MSAlign+ software<sup>44</sup> in HIC fraction 2. For example, LC/MS/MS identified a protein of 9.5 kDa with a RPC retention time of ~37 min as a subunit of HU transcriptional dual regulator protein with a total of 71 unique *b/y* fragment ions (Figure 6a). Similarly, a protein of 15.7 kDa at a RPC retention time of ~28 min is identified as superoxide dismutase precursor (Cu–Zn) protein with a total of 60 unique *b/y* ions and an N-terminal truncation between two alanine residues (Figure 6b). The fragment ions were isotopically resolved as exemplified by two fragment ions at *m/z* of 916 (inset of Figure 6a) and *m/z* of 2024 (inset of Figure 6b). Noteworthy is the unit mass resolution observed for

a larger fragment (e.g.,  $Y_{139}^{7+}$ ) of ~14.1 kDa at high mass (>2000  $m/z$ ) in a single MS scan on Q Exactive. Table S-1 lists the proteins with a MW up to 32.1 kDa identified from HIC fraction 2 using the HIC-RPC-MS sequence. The high numbers of matching fragments and significantly low  $P$ ,  $E$  values correlate statistically with higher confidence in identification. These results demonstrate that HIC can be effectively coupled with RPC and MS/MS for top-down proteomics.

## CONCLUSIONS

In summary, we have established a novel and effective HIC-RPC-MS combination which is promising for overcoming the bottleneck of separation that has hindered progress in top-down proteomics. Central to this success is the identification of ammonium tartrate as a salt that is compatible with MS (in terms of not interfering with downstream MS analysis). This overcomes the challenges encountered for the commonly used ammonium sulfate HIC buffer including significant sulfate adduction and concomitant suppression of the intact protein signal even after extensive desalting. We further demonstrate that the selectivity of HIC and RPC is orthogonal and can be utilized to significantly increase the separation power as demonstrated here for standard protein mixtures and a complex cell lysate. Hence, for the first time, we demonstrate the high potential of HIC in coupling with RPC for top-down proteomics.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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