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mass spectrometry**

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Effects of amino acid additives on protein solubility - insights from desorption and direct electrospray ionization mass spectrometry

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27 **Keywords:** Protein dissolution, Protein solubility, Amino acid additives, Serine, DESI-MS, ESI-
28 MS, Protein folding, Protein stability, Aggregation
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Abstract

Naturally occurring amino acids have been broadly used as additives to improve protein solubility and inhibit aggregation. In this study, improvements in protein signal intensity obtained with the addition of L-serine, and structural analogs, to the desorption electrospray ionization mass spectrometry (DESI-MS) spray solvent was measured. The results were interpreted at the hand of proposed mechanisms of solution additive effects on protein solubility and dissolution. DESI-MS allows for these processes to be studied efficiently using dilute concentrations of additives and small amounts of proteins, advantages that represent real benefits compared to classical methods of studying protein stability and aggregation. We show that serine significantly increases the protein signal in DESI-MS when native proteins are undergoing unfolding during the dissolution process with an acidic solvent system (p-value=0.0001), or with ammonium bicarbonate under denaturing conditions for proteins with high isoelectric points (p-value=0.001). We establish that a similar increase in the protein signal cannot be observed with direct ESI-MS and the observed increase is therefore not related to ionization processes or changes in the physical properties of the bulk solution. The importance of protein conformational changes during dissolution in the presence of serine is demonstrated through comparisons between the analyses of proteins deposited in native or unfolded states and by using native state-preserving and denaturing desorption solvents. We hypothesize that direct, non-covalent interactions involving all three functional groups of serine are involved in the beneficial effect on protein solubility and dissolution. Supporting evidence for a direct interaction include a reduction in efficacy with D-serine or the racemic mixture, indicating a non-bulk-solution physical property effect; insensitivity to the sample surface type or relative placement of serine addition; and a reduction in efficacy with any modifications to the serine structure, most notably the carboxyl functional group. An

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3 alternative hypothesis, also supported by some of our observations, could involve the role of serine
4 clusters in the mechanism of solubility enhancement. Our study demonstrates the capability of
5 DESI-MS together with complementary ESI-MS experiments as a novel tool for understanding
6 protein solubility and dissolution and investigating the mechanism of action for solubility-
7 enhancing additives.
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14 **Introduction**

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17 Understanding the mechanisms governing protein solubility and aggregation at the molecular
18 level is of great importance to many fields including biochemistry, pharmaceutical sciences, and
19 clinical studies.¹⁻³ Osmolytes are a diverse group of small molecules naturally selected to protect
20 proteins against different stress factors while maintaining protein function⁴⁻⁵ and have widespread
21 applications in many fields.⁶⁻⁷ Naturally occurring amino acids are amongst the osmolytes that
22 have been studied for nearly four decades⁸ for enhancing protein solubility and reducing protein
23 aggregation⁹⁻¹¹. The effect of arginine (Arg)¹² on suppressing protein aggregation¹³ and the
24 stabilizing effect of several other amino acids including glycine (Gly),¹⁴⁻¹⁵ proline (Pro),¹⁶⁻²⁰
25 histidine (His),²¹ alanine (Ala),²² glutamic acid (Glu) - arginine mixtures,²³ and small amines²⁴⁻²⁵
26 have demonstrated the ability of amino acid additives to stabilize proteins under stress conditions
27 and to improve protein solubility by suppressing aggregation.
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43 Despite decades of extensive studies, there is no unified theory that can explain how amino
44 acids inhibit protein aggregation²⁶. Progress has been made to successfully differentiate between
45 the effects of additives on native versus unfolded or denatured structures.²⁷ Some additives help
46 stabilize the structure of native proteins and therefore improve stability, whereas others may
47 destabilize the native structure, or show little effect on protein structure but effectively suppress
48 aggregation.²⁸⁻³² Amongst the proposed mechanisms, preferential interaction theory,³³⁻³⁶ crowding
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3 effects³⁷⁻³⁸ and/or the gap effect³⁹ have been widely studied. These proposed mechanisms have
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5 predominantly been examined or modeled at high concentrations of additives (in ≥ 50 mM range)
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7 in bulk solution by conventional biophysical techniques,^{31, 40-42} and have not yet been studied at
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9 micro-scale with techniques that can operate at lower quantities and concentrations.

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11 Desorption electrospray ionization mass spectrometry (DESI-MS) couples extractive-desorption
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13 of the samples with electrospray ionization.⁴³ The analysis of sample in DESI occurs via the
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15 droplet-pickup mechanism⁴⁴, where charged droplets of solvent impact the sample, and a thin,
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17 localized solvent layer is formed on the surface, dissolving the analytes. Progeny droplets
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19 containing the extracted analyte are formed via subsequent droplet collisions and undergo
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21 electrospray ionization processes.
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27 DESI provides similar spectral information of proteins to what is obtained with ESI-MS⁴⁵⁻⁴⁷ albeit
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29 at lower intensities, and the technique especially struggles with larger proteins.^{45, 48} Various
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31 approaches have been developed to improve DESI analysis of proteins. For example coupling ion
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33 mobility separation with DESI⁴⁹⁻⁵⁰, creating a very short sampling pathway⁵¹ and using a pre-
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35 wetting technique.⁵² By deconstructing DESI and independently investigating protein desorption
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37 and ionization, we confirmed that inefficient protein dissolution during the short time-scale of
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39 DESI is a major contributor to the lower protein signal.^{48, 53}
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43 Non-specific protein aggregates in concentrated solutions or droplets, such as those generated by
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45 electrospray are prevalent, and the formation of aggregates in both bulk solution and droplets due
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47 to weak non-covalent interactions is well-established.⁵⁴⁻⁵⁵ Aggregate numbers close to 20 were
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49 observed in the subpopulation of droplets with apparent diameters near 220 nm and protein
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51 concentration of 4 μM , and the probability of higher aggregate numbers increase with higher
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53 protein concentrations and larger droplet sizes.⁵⁶ Considering the thin, micro-localized solvent
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3 layer on the sample surface, into which proteins are dissolved, and the size of DESI progeny
4 droplets (average 1-4 μm)⁵⁷, aggregates can form extensively due to the high concentration of
5 unfolding protein, resulting in protein signal reduction. The solubility of proteins in the desorption
6 solvent and aggregate formation directly affects the efficacy of protein dissolution⁵⁸ in the
7 desorbing solvent during the short DESI time-frame,⁵⁹ which provides a reasonable explanation of
8 why the analysis of proteins by DESI-MS is inherently more difficult than small molecules.^{45, 48}

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10 Additives have been a convenient approach for improving protein solubility in DESI. We
11 previously explored approaches such as gas-phase additives, for example vapors of ethyl acetate,⁶⁰
12 and solution-phase additives such as ammonium bicarbonate⁶¹ and the amino acid L-serine⁶² in
13 order to enhance protein analysis by DESI-MS.

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15 Applications of amino acid additives in ESI-MS of proteins have been explored relatively recently.
16 The stabilizing effect of amino acids against thermal denaturation of non-covalent protein
17 complexes in ESI-MS was reported and contributed to ion ejection and reduced columbic
18 repulsion.⁶³ The stabilizing effect of amino acids and imidazole on protein-ligand ions against in-
19 source fragmentation during ESI-MS has also been studied and reported as highly dependent on
20 ESI conditions and instrumentation⁶⁴ and likely related to an evaporative cooling mechanism.⁶⁵

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22 It has been shown that amino acid additives, L-serine in particular, can improve protein analysis
23 by removing sodium adducts from high NaCl concentration in the protein sample during native
24 ESI⁶⁶ and DESI.⁶² Since DESI progeny droplets follow the same ionization process as ESI
25 droplets,⁴⁴ the desalting effect of L-serine, which was observed in both types of experiment, is
26 likely related to the electrospray ionization process. On the other hand, L-serine also increased
27 signal intensity of purified proteins in DESI-MS when combined with formic acid or ammonium
28 bicarbonate as co-additives, but no such effect was observed in ESI-MS of similar protein

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3 solutions, indicating that this effect is independent of electrospray ionization processes and related
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5 to protein dissolution and desorption.
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8 With DESI-MS and ESI-MS the analysis of proteins occurs via the same ionization mechanisms,
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10 therefore, relative changes in signal between these two techniques provide a window into studying
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12 protein dissolution and desorption.^{48, 53} With DESI, the short but controllable interaction time of
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14 proteins with denaturing solvents and additives before detection allows one the unique opportunity
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16 to study protein unfolding during dissolution, while in non-native ESI, unfolding has already
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18 occurred during sample preparation, long before protein detection. In this study we interpret
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20 relative changes in protein signal intensities as protein dissolution effects, after controlling for
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22 possible ionization differences using comparable experiments in ESI. We demonstrate that
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24 mechanistic insights into the effects of solution-phase additives on proteins can be obtained at
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26 much lower additive concentrations and protein amounts, compared to most biophysical
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28 techniques that are commonly used for this purpose.
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35 **Experimental**

36 **Materials**

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38 Lyophilized equine heart cytochrome *c* (Cyt *c*, 12.3 kDa, pI=10.3) and equine muscle myoglobin
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40 (Myo, 16.9 kDa, pI=7.4) were purchased from Sigma-Aldrich (St. Louis, MO). Proteins were at
41
42 $\geq 95\%$ purity and were used without further purification. BioUltra grade ammonium bicarbonate
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44 (ABC), ammonium acetate (NH₄OAc), A.C.S. grade ammonium hydroxide (NH₄OH), HPLC-MS
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46 grade methanol (MeOH), LC-MS grade formic acid (FA) and all amino acids ($\geq 98\%$) were
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48 purchased from Sigma-Aldrich (St. Louis, MO) except N-acetyl L-serine which was purchased
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50 from Carbosynth (Berkshire, UK). Milli Q water was obtained from a Thermo-Barnstead Water
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3 Polisher. Fused silica capillaries were purchased from Trajan Scientific (Ringwood, Australia).
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5 Porous polyethylene surfaces (PE) with average pore size of 15-45 μm (POREX-4900) were
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7 purchased from Interstate Specialty Products (Sutton, MA). PTFE plates were purchased from
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9 Prosolia Inc (Indianapolis, IN).
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11 **Sample preparation and solvent systems**

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14 Stock solutions of each protein were made by dissolving the lyophilized protein powder in Milli
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16 Q water to a final concentration of 800 μM or 80 μM depending on the desired surface
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18 concentration. Protein solutions were sprayed on the surface with a pneumatically-assisted
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20 nebulizer spray made of two coaxial fused silica capillaries similar to an ESSI sprayer.⁶⁷ The
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22 sprayer was orthogonally positioned at 3 mm above the surface. Nebulizing gas pressure and flow
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24 rate were 100 psi and 3 $\mu\text{l}/\text{min}$ respectively, and the stage was moved at 150 $\mu\text{m}/\text{s}$, resulting in
25
26 long homogenous protein lines deposited with 1.1 ± 0.1 mm widths. The average surface
27
28 concentration of these protein lines was approximately 22 pmol/mm^2 for all DESI experiments
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30 with the exception of spectra in Figure 1 and Figure S1-S2 which were obtained from protein lines
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32 with an approximate surface concentration of 10X higher (222 pmol/mm^2) in order to obtain clear
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34 and comparable spectra for all different solvent systems regardless of the inherently lower
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36 sensitivity of the non-acidic solvents. For PTFE slides, 3 μl volumes of 20 μM aqueous protein
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38 solutions were micro pipetted on the slide and dried under vacuum for 30 minutes at approximately
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40 85 kPa. The diameter of the dried spot was approximately 1.2 mm, resulting in average protein
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42 surface concentration of 24 pmol/mm^2 . ESI experiments used 10 μM protein in the appropriate
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44 solvent systems.
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51 All DESI desorption solvent systems were made in 50% MeOH:H₂O. Aqueous stock solutions
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53 of 2.0 M ammonium bicarbonate and 2.0 M ammonium acetate were used to prepare 200 mM
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3 dilutions in 50% MeOH (approximately pH 8.0 and 7.3, respectively). LC-MS grade formic acid
4 and ammonium hydroxide were used to prepare 0.1% (v/v) formic acid and 1% (v/v) ammonium
5 hydroxide in 50% MeOH (approximately pH 3.0 and 10, respectively). Serial dilutions from
6 aqueous amino acid stock solutions were used to make 100 μ M amino acid in the solvent system.
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8 The pH of aqueous solutions was measured at room temperature with a Mettler Toledo Seven Easy
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dilutions in 50% MeOH (approximately pH 8.0 and 7.3, respectively). LC-MS grade formic acid and ammonium hydroxide were used to prepare 0.1% (v/v) formic acid and 1% (v/v) ammonium hydroxide in 50% MeOH (approximately pH 3.0 and 10, respectively). Serial dilutions from aqueous amino acid stock solutions were used to make 100 μ M amino acid in the solvent system. The pH of aqueous solutions was measured at room temperature with a Mettler Toledo Seven Easy pH meter (Columbus, OH) equipped with an InLab Expert pH electrode. Similar to previous relevant studies,⁶⁸ the reported pH values were not corrected for the influence of MeOH as it was deemed inconsequential to the experiments.

Instrumentation and experimental parameters

A linear ion trap mass spectrometer, LTQ (Thermo Scientific, Waltham, MA, USA) was combined with a 3-dimensional translational stage (Purdue University, West Lafayette, IN, USA) for DESI analyses. An extended ion sampling capillary with a 5 cm extension was purchased from Scientific Instruments Inc. An electrospray emitter and desorption sprayer was prepared from a Swagelok T-piece and two pieces of coaxial fused silica capillary tubing.⁶⁷ The outer capillary (for sheath gas) was approximately 20 mm in length with an outer diameter of 430 μ m and inner diameter of 320 μ m. The internal capillary (for solvent) had an outer diameter of 220 μ m, and inner diameter of 50 μ m. The solvent capillary extended through the T-piece and was connected to a syringe pump which delivered the solvent.

DESI sprayer incident angle was 54°. The distance between the desorption sprayer and heated extended capillary was 4 mm. The tip of the desorption sprayer was 1 mm above the surface. ESI experiments were performed under the same conditions as DESI, but protein solutions were directly sprayed towards the heated extended capillary at 5 μ L/min with 100 psi N₂ using the same sprayer.

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3 Spray potential was set at 4.0 kV and was applied to the liquid junction of a stainless-steel syringe
4 needle which delivered solvent at a flow rate of 5 $\mu\text{L}/\text{min}$, with N_2 as nebulizing gas at 100 psi.
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6 Capillary temperature was set at 250°C. LTQ ion optic parameters were optimized by LTQ
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8 TunePlus automatic tuning feature for each protein's highest intensity charge state (HICS) peak,
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10 using direct infusion of 5 μM protein in each solvent system.
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14 **Data Analysis**

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17 Mass spectra were collected and viewed in Xcalibur Qual Browser (2.0.7). At least three
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19 independent trials were collected for each solvent system. In each trial three protein sample lines
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21 or spots were perpendicularly scanned and averaged. Signal intensities were calculated as the
22
23 average of three trials and error bars represent \pm mean standard deviation. MagTran software (1.03)
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25 was used for charge state deconvolution and calculation of integrated protein signal intensity and
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27 S/N as described by Zhang and Marshall.⁶⁹
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33 **Results and discussion**

34 **Influence of solvent composition on protein signal increase by L-serine**

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36 Previously, we showed that the addition of low millimolar L-serine concentrations into 50%
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38 MeOH:H₂O desorption solvent can improve sensitivity for protein detection by DESI-MS in the
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40 presence of 1 mM and higher concentrations of NaCl,⁶² similar to observations reported in native
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42 ESI-MS experiments.⁶⁶ However, L-serine also increased signal intensity of purified proteins in
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44 DESI-MS when combined with formic acid or ammonium bicarbonate as co-additives, but no such
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46 effect was observed in ESI-MS of similar protein solutions. To investigate the role of solvent
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48 composition on the mechanism governing increased protein signal in DESI-MS with addition of
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50 L-serine, the combination of L-serine with the following four solvent systems were compared:
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3 formic acid, ammonium acetate, ammonium bicarbonate, and ammonium hydroxide. All solvents
4 were made in 50% MeOH:H₂O and therefore 50% MeOH:H₂O was treated as additive-free
5 control. Out of the five different solvent systems, only the formic acid containing solution showed
6 an increase in protein signal with L-serine addition for both cytochrome *c* and myoglobin.
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8 However, L-serine was also able to increase the signal intensity for cytochrome *c* in ammonium
9 bicarbonate containing solutions, as seen in Figure 1. A complete set of representative spectra for
10 all five solvent systems, and changes in protein signal intensities with L-serine addition, can be
11 found in Figure S1 and Table S1.
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21 An important consequence of changing solvent additives is the solution pH and the effect this can
22 have on the protonation state of sample species. Serine was added into solvent systems that
23 spanned a pH range of 3 to 10. The estimated protonation populations of serine and the protein net
24 charge in the different pH ranges can be found in Table S2. Although the solution pH can affect
25 serine protonation state, and therefore population ratios of different serine species in the solution,
26 in all solvent systems except ammonium hydroxide, zwitterionic serine is the predominant form.
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28 No correlation between desorption solvent pH and increased protein signal intensities with L-
29 serine was observed.
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40 The other important consequence of changing solvent composition is protein conformational
41 changes. Lower charge states, indicative of more folded protein conformations, were observed
42 with desorbing solvents containing 50% MeOH, and when ammonium acetate or ammonium
43 hydroxide was added into this solvent (Figure S1). In ammonium acetate and ammonium
44 hydroxide the change in protein signal intensities with serine addition were not statistically
45 significant (p -value>0.05) and in the additive-free solvent system the signal even deteriorated.
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3 nonspecific adducts and spreading of the protein signal over multiple peaks which causes a
4 reduction in the signal intensity of the highest intensity charge state (HICS). As can be observed
5 in Figure S1 (d) and (f) for myoglobin, the extensive overlapping peaks resemble non-specific
6 adduction of small molecules to proteins⁷⁰⁻⁷¹.
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11 In contrast, protein signal intensities increased by a factor of 3.8 ± 0.1 for cytochrome *c*, and by a
12 factor of 4.3 ± 0.3 and 3.5 ± 0.2 for apo-myoglobin and holo-myoglobin, respectively, with addition
13 of L-serine to the desorption solvent containing 0.1% formic acid (Figure 1 (c,d)). Similarly, with
14 the addition of L-serine to 200 mM ammonium bicarbonate as desorption solvent, cytochrome *c*
15 signal intensity increased by a factor of 3.1 ± 0.2 (Figure 1 (e,f)). The signal intensity for
16 myoglobin, on the other hand, signal increased only by a factor of 1.2 ± 0.2 (Figure 1 (g,h)), which
17 was not statistically significant (p -value >0.05). Extensive unfolding was also observed for both
18 proteins, despite the near-neutral pH of the solvent system and buffering by ammonium
19 bicarbonate. It was previously proposed that protein unfolding with ammonium bicarbonate
20 containing solutions during ESI is likely due to protein destabilization inside the heated
21 electrospray droplets, either because of an increase in hydrophobic surface area by bubble
22 formation, or due to electrothermal supercharging.⁷²⁻⁷³
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40 A comparison between the two proteins suggests that this difference could be related to protein
41 net charge in solution. Cytochrome *c* and myoglobin are both single-chain peptides of similar size
42 (12.3 kDa and 16.9 kDa respectively), but their isoelectric points are approximately 10.3⁷⁴ and
43 7.4⁷⁵, respectively. This difference in isoelectric points results in myoglobin having a negative to
44 near neutral net charge in ammonium bicarbonate solution, whereas cytochrome *c* has a positive
45 net charge. Likewise, protein net charge is an important factor in determining the effect of
46 Hofmeister series and osmolytes on protein solubility.⁷⁶⁻⁷⁷
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3 Therefore, as evident from a comparison between the behaviors of the two proteins in the four
4 desorbing solvent systems, a denaturing desorbing solvent and protein with a net positive charge
5 are key factors in the mechanism governing the substantial improvement in protein signal with L-
6 serine.
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11 **Role of protein conformational change and serine addition timepoint**

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15 To investigate the role of protein conformation prior to the interaction with L-serine, cytochrome
16 *c* and myoglobin were spray-deposited out of aqueous solution (native-like conformation) or out
17 of 0.1% formic acid solution (unfolded conformation) and analyzed with a desorption spray
18 solvent containing 0.1% formic acid in 50% MeOH in the presence or absence of 100 μ M L-serine.
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26 As seen in Figure 2, for both cytochrome *c* and myoglobin signal intensities of proteins deposited
27 in native conformation (top panel) increased more with serine addition than proteins already
28 unfolded (bottom panel). The signal intensity for natively deposited cytochrome *c* and apo-
29 myoglobin increased by a factor of 2.6 ± 0.3 and 3.8 ± 0.8 , respectively. Despite using 50% MeOH
30 and formic acid as the desorbing spray solvent, holo-myoglobin signal was also observed for the
31 natively deposited sample and increased by a factor of 3.5 ± 0.2 with serine addition, as seen in
32 Figure 2 (b). When the samples were deposited out of denaturing solution, serine addition achieved
33 only mild increases in signal of 1.3 ± 0.3 and 1.5 ± 0.4 for cytochrome *c* and apo-myoglobin with *p*-
34 values 0.098 and 0.042, respectively (Figure 2 (g,h)).
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47 In the previous section we showed the importance of denaturing solvent systems for the beneficial
48 effect of serine addition on signal intensities. The difference in relative improvements between
49 native and unfolded protein prior to analysis further indicates that serine should be present during
50 the unfolding process.
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3 A similar increase in signal intensity does not occur under denaturing conditions upon the addition
4 of L-serine when proteins are analyzed in solution using direct ESI infusion.⁶² Figure 3 shows 10
5 μM of each protein, electrosprayed out of the exact solvent systems as for the DESI results in
6 Figure 1 (a) and (b) and Figure 2 (0.1% formic acid in 50%MeOH). The order of adding formic
7 acid and L-serine was also alternated to measure the effect of unfolding in bulk solution in the
8 presence of L-serine. Addition of 100 μM L-serine to the working solution before and after protein
9 unfolding through acidification made no meaningful difference in protein ESI signal intensity,
10 although the changes in absolute signal intensity between adding serine first was statistically
11 significant (p -value<0.05) for cytochrome *c*. Adding L-serine prior to acidification in bulk solution
12 increased the signal for cytochrome *c* by a mere 1.3 times compared to when it was not present (p -
13 value=0.001). On the other hand, when serine was added after formic acid addition to the
14 cytochrome *c* solution, there was no statistically significant change compared to not having serine
15 in the solution at all (p -value>0.05). In the case of myoglobin, addition of serine reduced the
16 absolute signal intensity compared to no serine present, and there was no statistical difference
17 between adding serine before or after formic acid (p -value=0.56), as seen in Figure 3. The lack of
18 improvement in protein signal intensity in ESI reiterates that the improvement in protein signal
19 intensity in DESI is likely related to the dissolution of proteins during desorption. We have
20 previously shown that proteins desorb equally well from the surface in DESI, and poor dissolution,
21 rather than physical desorption or ionization problems are the major contributor to poor DESI-MS
22 signals for protein.^{48, 53} The lack of improvement in protein signal with L-serine in ESI (Figure 3)
23 and the difference between timescales of unfolding and dissolution to ion detection in DESI versus
24 ESI possibly point towards affecting the kinetics of dissolution, rather than a thermodynamic effect
25 on solubility. These observations together imply that dissolution of native proteins in denaturing
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3 solvents are positively influenced in the presence of L-serine as a solution additive, manifested by
4 an increase in protein signal intensity measured by DESI-MS.
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10 **Relating DESI observations with known models for suppression of protein aggregation by** 11 **amino acids** 12

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14 One of the most consequential repercussions of protein unfolding is aggregation. Mechanisms of
15 protein unfolding and subsequent aggregation can involve non-covalent weak interactions such as
16 hydrophobic and electrostatic interactions, hydrogen bonding, Van der Waals interactions, or
17 covalent disulfide bonds.⁷⁸ Many studies support the proposed mechanism that amino acid
18 additives can improve protein solubility by suppressing aggregation of unfolded species or
19 partially unfolded intermediates.⁷⁹⁻⁸⁰ This has been demonstrated using in-vitro and in-silico
20 studies, by NMR, X-ray spectroscopy, and crystallography, often implicating direct binding to
21 folding intermediates.^{12, 41, 81-83}
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33 The exact mechanism of action for the prevention of aggregation by additives including amino
34 acids is yet under debate,⁸⁴ but here we place our observations from DESI-MS and ESI-MS
35 experiments into context of two widely studied theories on additive effects on protein solubility.
36 *Preferential interaction theory*³³⁻³⁶ measures changes in thermodynamic properties such as
37 interaction of protein surface with additive and water in bulk solution. Based on this model,
38 additives that stabilize protein such as most osmolytes, including a number of amino acids, are
39 preferentially excluded from the protein surface and can influence protein folding equilibrium.⁸⁵
40 Other additives, such as urea and arginine that are not excluded from the surface of the protein and
41 are weakly bound, do not stabilize native protein structure, but improve protein solubility through
42 aggregation suppression.⁸⁵⁻⁸⁶ In our data, L-serine did not shift the protein charge state distribution
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3 to those representing more folded charge states, and also destabilized myoglobin (Figure 2). Chen
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5 *et al.* have reported similar observations in ESI-MS of myoglobin in 20 mM ammonium acetate
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7 buffer with L-serine.⁶³ Based on these observations and according to the preferential interaction
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9 theory, we propose that L-serine behaves as a destabilizing additive, and similar to arginine, binds
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11 to the protein through weak interactions to suppress protein aggregation.
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17 *The gap effect theory* differentiates between reversible or irreversible aggregation and non-native
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19 or native aggregation and has been proposed based on results from computational models and
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21 molecular dynamic simulations.³⁹ Based on this model, as two protein molecules associate, a gap
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23 is formed in which an amino acid additive⁸⁷ is considered neither preferentially bound nor
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25 completely excluded from the protein surface (known as a neutral crowder), while allowing water
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27 molecules to interact with proteins. Such additives increase the free energy of protein–protein
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29 molecules to interact with proteins. Such additives increase the free energy of protein–protein
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31 association and slow aggregation, not because of changes in thermodynamic properties but based
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33 on a kinetic effect on protein association during aggregation. Comparison between Figure 2 and
34
35 Figure 3 supports the hypothesis that L-serine is perhaps affecting the kinetics of protein
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37 aggregation during unfolding which can be captured due to the short timescale of protein
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39 dissolution in DESI compared to the time between sample preparation and analysis by direct ESI.
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41 Recent studies have reported more evidence for direct interaction between amino acid additives
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43 and protein^{42, 88-89} including FTIR spectroscopy and molecular dynamic simulations that show a
44
45 direct interaction between proline and lysozyme⁴² and X-ray crystallography study that show
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47 binding of glycine amide to hydrophobic residues of lysozyme.⁸¹ Non-covalent binding of multiple
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49 amino acids to native and denatured cytochrome *c* in ESI-MS has also been reported.⁹⁰ It was
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51 proposed that the formation of stable non-covalent complexes with these amino acids depend on
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3 stable ionic interactions and successful formation of hydrogen bonds with specific charged
4 residues of the protein, especially those with the least steric and electrostatic repulsion between
5 amino acid additive and the protein. We also observed L-serine adducts with $S/N > 3$ on charge
6 states +7 to +12 at low temperatures (70°C) in both ESI-MS and DESI-MS (Figure S2). The
7 presence of L-serine adducts on protein peaks in the denaturing solvent 0.1% formic acid in 50%
8 MeOH provides evidence that interactions between unfolding protein and L-serine is possible. In
9 the following sections, we further explore the possible direct interactions between L-serine
10 functional groups and the protein.
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24 **Investigating serine-surface interactions involved in mechanism of signal enhancement**

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26 During the unfolding process, it is possible that the denaturing protein can bind more tightly with
27 the surface as well as with other proteins in solution, due to exposure of a larger surface area of
28 either hydrophobic or polar amino acids from the protein core. To evaluate the potential role of
29 serine disrupting protein-surface interactions, the more hydrophilic PE surface, that was used for
30 the data presented in Figure 2 (a), was replaced with the more hydrophobic surface of porous PTFE
31 which has a different polarity and dielectric constant. As shown in Figure 4 (a), changing the
32 identity of the surface to the more hydrophobic PTFE did not influence the extent of protein signal
33 increase with the addition of L-serine to the solvent.
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44 Additional evidence for eliminating surface interactions from contributing to the beneficial L-
45 serine effect is demonstrated in Figure 4 (b). To evaluate the potential for L-serine to disrupt
46 protein surface interactions, L-serine was added to the surface before protein deposition, after
47 protein deposition and premixed into protein stock solution. As demonstrated in Figure 4 (b), there
48 was no statistical difference between these different methods of serine application (p -value > 0.05),
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3 although it could be argued that there is considerable mixing occurring on the sample surface
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5 during the turbulent DESI analysis. Based on these observations, it is unlikely that protein-surface
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7 interactions play a dominant role in the beneficial effect serine has on protein signal with DESI-
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9 MS. Moreover, since similar improvements were observed with serine either in the desorbing spray
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11 or when applied to the sample prior to desorption confirms that the improvement in signal intensity
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13 is not due to changes in physical parameters, such as surface tension of the bulk desorption solvent.
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17 Curiously, having L-serine in the aqueous protein stock solution prior to sample deposition did
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19 not yield any improvement in protein signal intensity. When L-serine is mixed with the protein
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21 stock solution prior to the analysis, similar to ESI experiments, L-serine comes into contact with
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23 protein in bulk solution (fully hydrated protein), whereas for every other sample in Figure 4 (b),
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25 the L-serine point of contact with protein starts with the dried protein dissolving into the desorption
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27 spray micro-localized layer (during the hydration process). The observations from Figure 4 (b)
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29 suggest that interaction between L-serine and the unfolding protein during the dissolution process
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31 results in the observed increases in protein signal intensity.
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38 **Investigating possible intermolecular interactions of serine with protein during solvation of** 39 40 **unfolding protein**

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42 After eliminating the possibility of L-serine interrupting protein-surface interactions, we
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44 investigated L-serine-protein interactions through modifying L-serine structure, including
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46 stereochemistry of the side chain and blocking the functional groups, as well as comparisons
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48 between similar L-amino acids with the hydroxyl group side chain.
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51 The least benign modification could be changing the L enantiomer to D. As Shown in Figure 5,
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53 D-serine is not as effective as L-serine in improving protein signal and the racemic mixture of
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3 serine was only slightly more effective than D-serine. This observation again suggests that the
4 improvement in protein signal is not due to changes in the physical properties of the solvent such
5 as surface tension, but perhaps the consequence of direct interactions between the additive and
6 protein. The role of stereoisomer also implies that a chiral interaction is part of the interaction
7 between protein and L-serine. Chiral recognition has been shown to play a role in weak interactions
8 and hydrogen bonding⁹¹ and also with chiral solute-water interaction in solutions.⁹² The chirality
9 of L-serine can potentially affect the hydrogen bonding, especially with the peptide backbone of
10 the protein which has the greatest potential for hydrogen bonding and is sterically influenced by
11 the chirality of the amino acids.⁹³

12 Surprisingly the improvement in signal with a racemic mixture of serine was no more effective
13 than D-serine, indicating that perhaps higher-order serine clusters are involved in the effect. Some
14 precedent has been set for the role of clusters of both arginine and proline as protein aggregation
15 suppressors.^{82, 94} It is a possibility that L-serine clusters can affect protein aggregation through
16 modulating protein-protein interactions in a similar manner. Although in our experiments the peak
17 intensities of these higher-order serine clusters were not dominant, it is fascinating that serine, to
18 an extent not reached by any other amino acid, forms unusually stable clusters, in particular serine
19 octamer, with a remarkable preference for homochirality.^{95 96} Recent studies have validated that
20 serine clusters indeed exist not only in the gas-phase, but also in solution, although at low
21 concentrations.⁹⁷⁻⁹⁸

22 In Figure 6, the importance of each functional group on L-serine in its potential direct interaction
23 with the unfolding proteins was investigated. We systematically modified the three functional
24 groups on the serine molecular structure and compared the effects on protein analysis of these
25 derivatives to L-serine. For both cytochrome *c* and myoglobin, any modifications on L-serine
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3 molecular structure resulted in a reduction of improvement in protein signal. Removing the
4 hydroxyl group from L-serine (by using L-alanine) had the least deleterious effect on signal
5 enhancement, and still improved the signal intensity, albeit not as effectively as L-serine. The
6 strongest deleterious effect was observed with blocking the carboxylate group, noting the
7 importance of this functional group in the interactions between L-serine and protein. Similarly,
8 FTIR and molecular dynamic simulations have also reported that the carboxyl group of proline
9 plays a dominant role in direct proline-protein interactions.⁴² Earlier we demonstrated that L-serine
10 is much more effective in improving protein signal intensity of unfolding protein when the protein
11 has a net positive charge (Table S2). One could speculate that the interaction between the
12 negatively charged carboxyl group on the zwitterionic serine and positive charges on the protein
13 contributes to the mechanism of protein dissolution improvement.
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28 The observation that all three functional groups of L-serine are important in the beneficial
29 interaction with protein during dissolution can support one of two hypotheses: Zwitterionic L-
30 serine could potentially have a direct interaction that involves all 3 functional groups interacting
31 with positively charged unfolding proteins; Alternatively, homochiral serine clusters might be at
32 play, as evidenced by lack of efficacy of racemic serine mixtures, and the potential for any
33 structural changes to also affect clustering. To elucidate the exact points of interactions on protein,
34 detailed investigations with complementary techniques such as molecular modeling is needed.
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44 **Conclusion**

45 We demonstrated the application of DESI-MS combined with ESI-MS as a novel approach for
46 probing the mechanism of solubility-enhancing additives such as amino acids by using L-serine as
47 a model additive. The effects of L-serine on signal intensity were investigated by using five
48 different native-state preserving and denaturing solvent systems, changing the protein
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3 conformation prior to interaction with the additive, and measuring changes in protein signal during
4 unfolding in the bulk solution before and after addition of L-serine, versus unfolding during
5 dissolution. These results were interpreted at the hand of existing models of amino acid action on
6 protein solubility. Our results indicate that protein-surface interaction interruption is unlikely to
7 contribute to improvements in protein signal enhancement. We hypothesize that L-serine
8 potentially influences protein-protein interactions by acting as a destabilizing neutral crowder and
9 by suppressing aggregation when it is present during unfolding of proteins carrying a net positive
10 charge in solution. Our observations could be explained by a possible direct, noncovalent, chiral,
11 three-pronged interaction between L-serine and the protein. Alternatively, it is possible that serine
12 clusters could be involved in the dissolution enhancing effect.
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26 DESI-MS studies provide a novel perspective for understanding the mechanisms governing the
27 effects of additives on protein dissolution, solubility, and aggregation. Complementary DESI-MS
28 and direct ESI-MS experiments under controlled conditions allow differentiating between
29 thermodynamic and kinetic effects of additives on solubility and dissolution. Integration of other
30 complimentary tools such as ion mobility and delayed desorption with DESI-MS will enable time-
31 resolved analysis of protein dissolution processes and aggregate measurement in the presence of
32 additives. In addition to providing new insights into mechanisms of different additive effects, even
33 at low concentrations, this novel perspective can be a useful tool for rapid development of additives
34 important for protein chemistry and its applications, such as protein therapeutics and formulation.
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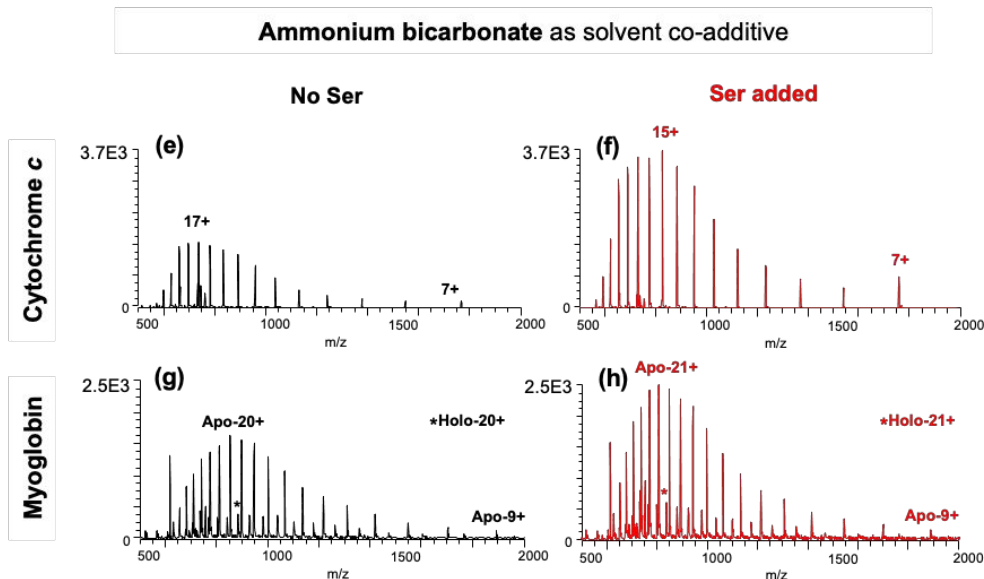
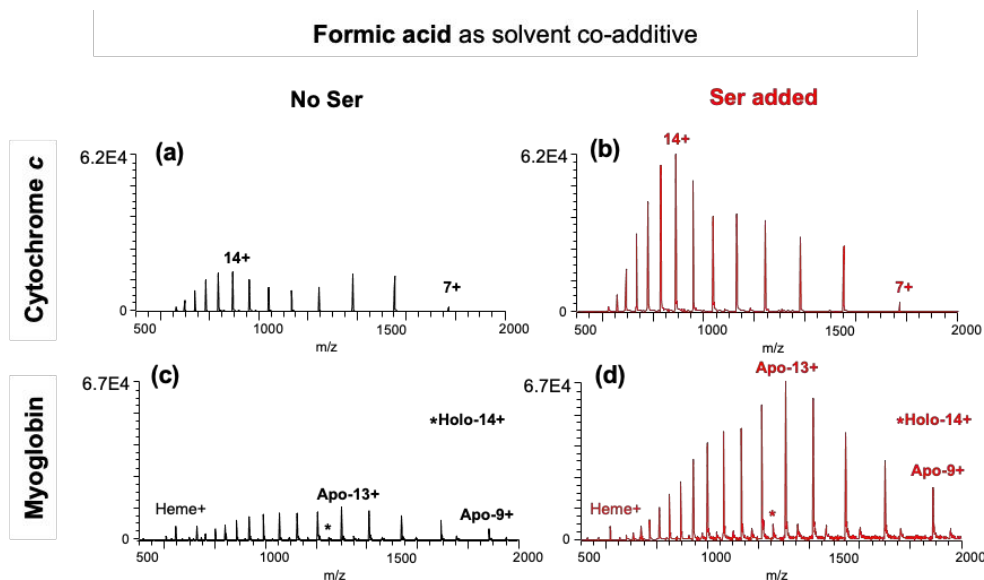


Figure 1. Representative DESI-MS spectra of natively deposited cytochrome *c* and myoglobin analyzed without L-serine (shown in black) and with 100 μ M L-serine (shown in red). (a), (c) 0.1% formic acid in 50% MeOH without L-serine, and (b), (d) with 100 μ M L-serine. (e), (g) 200 mM ammonium bicarbonate in 50% MeOH without L-serine, and (g), (h) with 100 μ M L-serine. The absolute intensities reported are the average intensity of the highest observed charge state (HICS) for the protein in each desorbing solvent system.

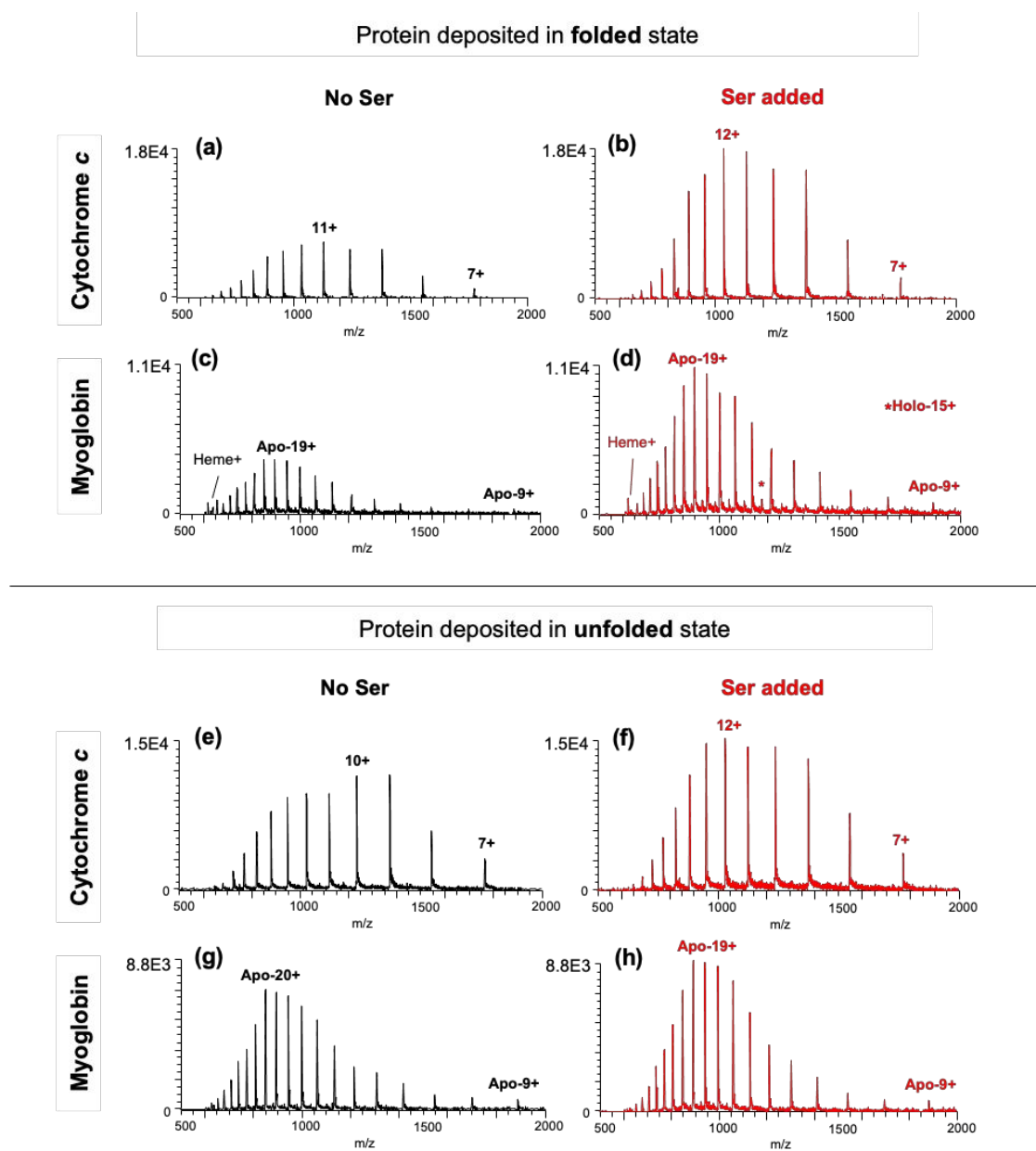


Figure 2. Representative DESI-MS spectra of cytochrome *c* and myoglobin deposited from (a-d) aqueous solution vs. (e-h) acidic solution, analyzed with 0.1% formic acid in 50%MeOH (shown in black) and with 100 μ M L-serine added (shown in red). The intensities are scaled to the average intensity of highest observed charge state (HICS) for each protein when analyzed with 100 μ M L-serine. This signal change was not statistically significant for denatured-deposited cytochrome *c*

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(p-value>0.05) and only marginally significant for denatured-deposited myoglobin (p-value 0.0420).

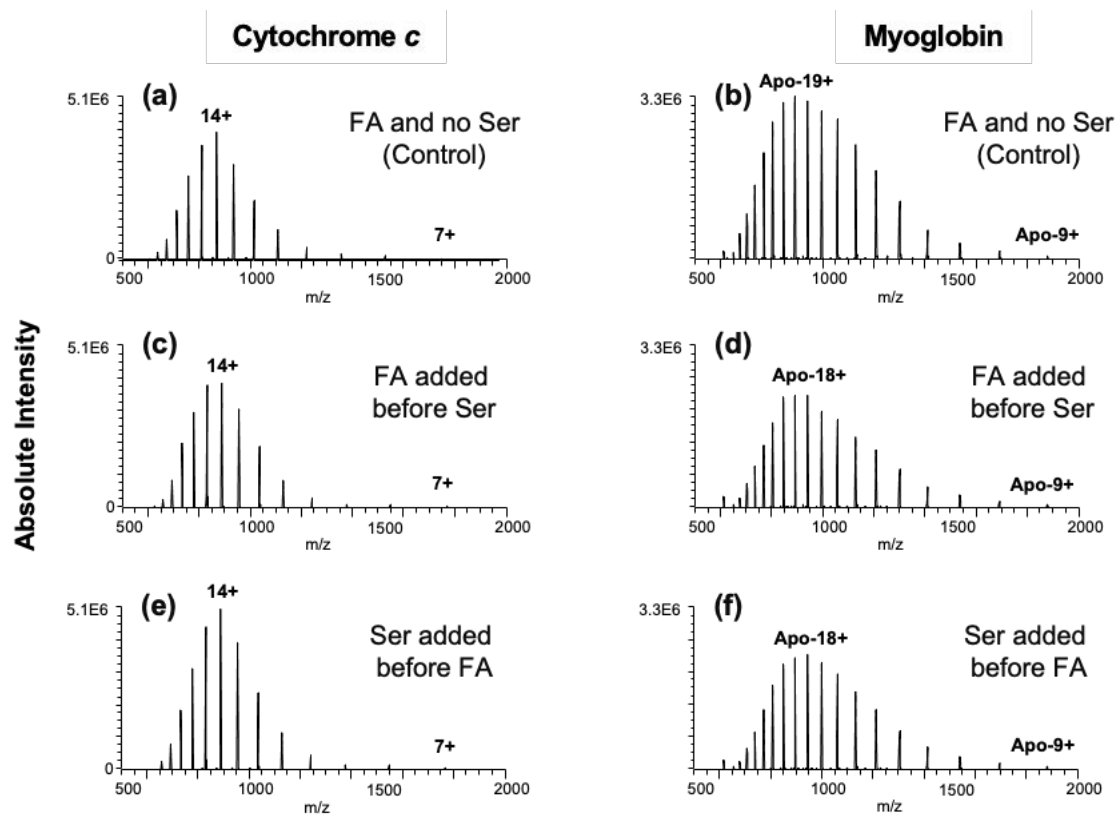


Figure 3. Representative ESI spectra of cytochrome *c* and myoglobin unfolding in bulk solution containing (a), (b) 0.1% formic acid only, (c), (d) containing 0.1% formic acid and 100 μ M L-serine with formic acid added first followed by serine after mixing to the protein solution, and (e), (f) containing 0.1% formic acid and 100 μ M L-serine with L-serine added first to the protein solution followed by formic acid.

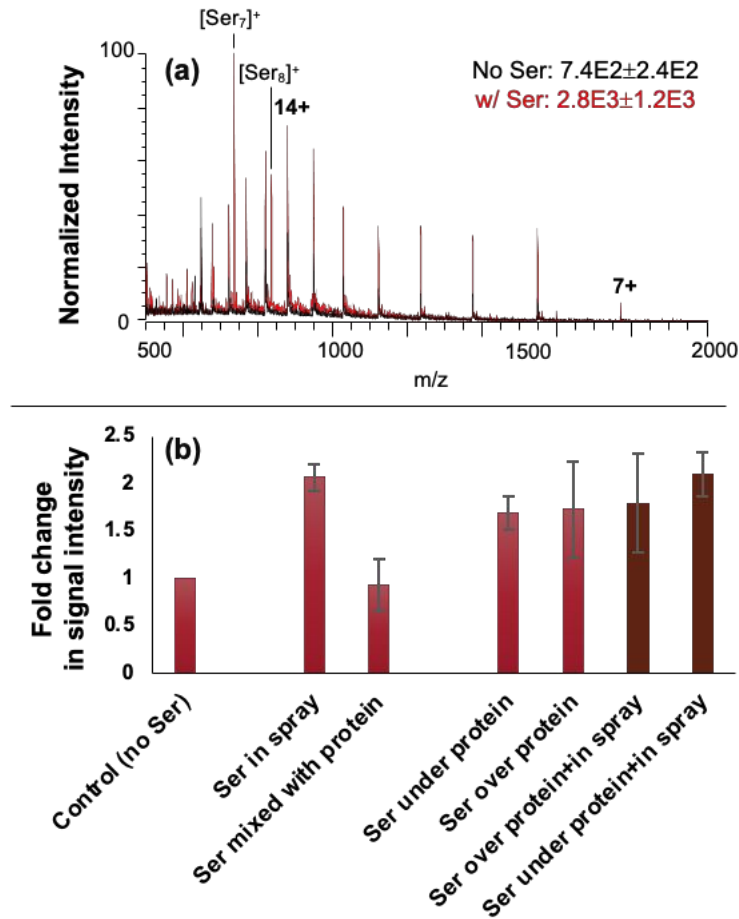


Figure 4. (a) Representative DESI-MS spectra of aqueous cytochrome *c* deposited on PTFE slides and analyzed with 0.1% formic acid in 50% MeOH (shown in black), and with 100 μ M L-serine (shown in red). The reported intensities are the average intensity of the highest intensity charge state (HICS) for cytochrome *c*. The equivalent PE data is found earlier in Figure 2 (a). (b) Signal intensity of cytochrome *c* on PE analyzed with 0.1% formic acid in 50% MeOH (control) and 100 μ M L-serine in different places during the DESI-MS analysis.

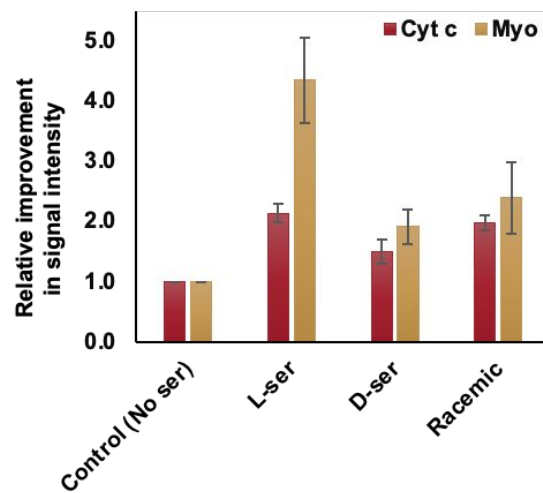


Figure 5. Comparison between serine enantiomers and the racemic mixture when used as solvent additives for analysis of natively deposited proteins with 0.1% formic acid in 50% MeOH.

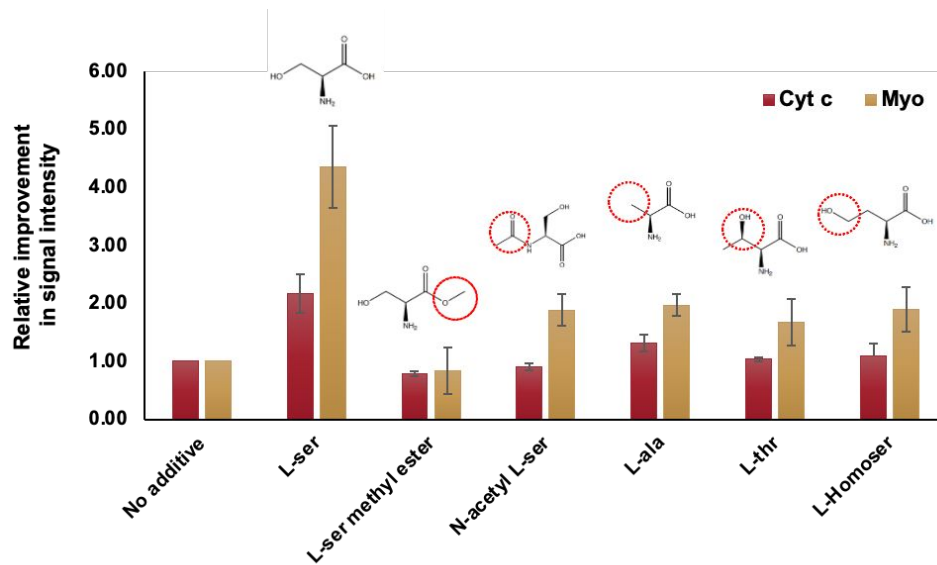


Figure 6. L-serine derivatives with systematically altered functional groups as additives and their effect on signal intensities of natively deposited cytochrome *c* and myoglobin with 0.1% formic acid in 50% MeOH. The modifications are highlighted in red circles and include methylation of the hydroxyl group, amidification of the primary amine of serine, a secondary versus primary hydroxyl or amine, and moving the hydroxyl group further away.