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1 **Purification of recombinant α-Synuclein: a comparison of commonly used protocols**

2 Amberley D. Stephens[1*], Dijana Matak-Vinkovic[2], Gabriele S. Kaminski Schierle[1*]

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4 [1]Chemical Engineering and Biotechnology, University of Cambridge, Philippa Fawcett Drive,
5 Cambridge, CB3 0AS

6 [2]Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW

7 *Corresponding author: asds2@cam.ac.uk, gsk20@cam.ac.uk

8

9 **Abstract**

10 The insoluble aggregated form of the protein alpha-synuclein (aSyn) is associated with
11 synucleinopathies, such as Parkinson's Disease, therefore great effort is put into understanding why
12 and how this initially soluble protein misfolds. The initial state of aSyn, e.g. presence of
13 contaminants, adducts, oligomers or degradation products, can greatly influence the outcome of an
14 assay, such as determining its aggregation kinetics. Here, we compare four commonly used protocols
15 for the isolation of recombinant aSyn from *E. coli* by boiling, acid precipitation, ammonium sulphate
16 precipitation and periplasmic lysis followed by ion exchange chromatography and gel filtration. We
17 identified, using non-denaturing electrospray ionisation mass spectrometry of the differently
18 extracted aSyn samples, that aSyn isolated by acid precipitation and periplasmic lysis yielded the
19 highest percentage of monomer, 100% and 96.5% respectively. aSyn purity was again highest in
20 samples isolated by acid precipitation and periplasmic lysis, yet aggregation assays displayed
21 differences in the aggregation rate of aSyn isolated by all four methods.

22 **Key words**: amyloid, hydrophobic interaction chromatography, native mass spectrometry,
23 intrinsically disordered protein

24

25 **Highlights**

26 - A rapid protocol; expression day one, two step purification day two.
27 - The periplasmic lysis-based protocol yielded 95% pure aSyn.
28 - Acid precipitation and periplasmic lysis-based protocols yielded the highest proportion of
29 monomeric aSyn at 100% and 96.5%, respectively.

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30 **Introduction**

31 The protein alpha-synuclein (aSyn) is the predominant protein found in insoluble aggregates called
32 Lewy bodies and Lewy neurites in the neurons of patients suffering from synucleinopathies, such as
33 Parkinson's Disease. Major emphasis has been put into studying how this usually soluble and
34 intrinsically disordered protein (IDP) misfolds into highly structured β-sheet containing fibrils. Many
35 studies have used purified recombinant aSyn to investigate its misfolding and cellular toxicity in both
36 in vitro and in vivo experiments. However, there are currently several different recombinant aSyn
37 purification protocols in use in the literature, many of which lack validation and quality control of the
38 recombinant protein used.

39 As an IDP, it is possible to separate aSyn from other proteins using methods to precipitate structured
40 proteins. By boiling a sample, many proteins undergo heat denaturation where internal bonds
41 become broken, disrupting the structure of structured/globular proteins and leading to
42 precipitation, yet heating leaves IDPs in solution due to their lack of structure. Acid shock works in a
43 similar way by disrupting intramolecular bonds but by altering the charge of the amino acids which
44 leads to altered bonding in the proteins and subsequent precipitation. Ammonium sulphate
45 ($(NH_4)_2SO_4$) precipitation is also used to purify aSyn. Increasing the percentage of $(NH_4)_2SO_4$ in
46 solution leads to precipitation of different proteins at different concentrations of $(NH_4)_2SO_4$ due to
47 favouring hydrophobic interactions and self-association[1]. Periplasmic lysis of aSyn from *E. coli* is a
48 more recent protocol that has been chosen as it is less 'harsh' on the protein compared to heating or
49 acid shock. aSyn is naturally trafficked to the periplasm when expressed in *E. coli*. To release aSyn,
50 but not the whole cell contents, the periplasm is lysed through osmotic stress [2].

51 aSyn resides as a dynamic ensemble of conformations in its soluble form and is therefore very
52 sensitive to its surrounding environment[3,4]. It is currently unclear whether the methods employed to
53 purify aSyn can actually influence the conformations formed within the dynamic ensemble or can
54 skew the ensemble and whether they can affect which aggregation-prone or non-aggregation-prone
55 pathways aSyn monomers will take. A few studies have investigated the effect of some isolation
56 treatments on the final recombinant aSyn, two studies investigating the effect of heating on the
57 structure of aSyn showed the presence of C-terminus truncated species after heating the protein to
58 95-100°C[5,6]. Yet, they reported no differences in the overall structure of full length aSyn by far-
59 ultraviolet circular dichroism (UV-CD) and nuclear magnetic resonance (NMR) spectroscopy[5]. A
60 purification method using acidification to precipitate unwanted proteins was the favoured method
61 by Giehm, et al.,[6] and was shown to give a higher purity of aSyn compared to boiling[7]. However, it
62 has been observed that acidification leads to C-terminus charge collapse and alteration of long-range

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63 interactions within the aSyn monomer, although again this was observed to be reversible[8,9]. It is
64 currently not clear whether 'reversible' changes to the conformation of aSyn actually do disrupt
65 intramolecular bonding and lead to small shifts in the dynamic ensemble of the aSyn conformations,
66 which are not detected by averaging measurements such as NMR or CD, or whether they can
67 influence aggregation rates and/or fibril polymorphism. We have previously shown, using the highly
68 sensitive technique of hydrogen-deuterium exchange mass spectrometry (HDX-MS), that the
69 method of storage does impact the monomeric aSyn structure. Lyophilisation, a commonly used
70 storage method for aSyn, leads to a compaction of aSyn monomers in comparison to freezing, even
71 when reconstituted in buffer. The compaction was not detected in methods such as dynamic light
72 scattering[10]. Lyophilisation leads to the formation of lyophilisation-induced oligomers, which were
73 different in structure to those in the frozen aSyn sample, and an increase in variability during ThT-
74 based aggregation assays. Therefore, the treatment of aSyn prior to experiments could be crucial in
75 terms of interpreting experimental data.

76 Here, we present a comparison of four aSyn isolation methods, boiling, acid precipitation (ppt),
77 $(NH_4)_2SO_4$ ppt and periplasmic lysis followed by ion exchange chromatography and gel filtration to
78 investigate the purity, proportion of monomer, aggregation rate and fibril polymorphs of aSyn
79 formed.

80

81 **Methods and Materials**

82 ***E. coli* expression of recombinant aSyn**

83 The plasmid pT7-7 containing human aSyn cDNA was transformed into *Escherichia coli* One Shot®
84 BL21 (DE3) Star™ (Thermo Fisher Scientific, USA). 0.5 L cultures of *E. coli* in Lysogeny Broth (LB)
85 containing carbenicillin (100 µg/mL) were grown at 37°C with shaking at 200 rpm and induced for
86 expression of aSyn when the OD_{600} reached 0.6-0.8 with 1 mM isopropyl-β-thiogalactopyranoside
87 (IPTG). After four hours of aSyn expression the cells were pelleted by centrifugation at 8 k x g for 15
88 mins.

89 **Preparation of protein samples for chromatography**

90 **Precipitation:** For acid ppt and $(NH_4)_2SO_4$ ppt methods, first the *E. coli* pellet from 500 mL of culture
91 were resuspended in 50 mL of lysis buffer (10 mM Tris, 1 mM EDTA pH 7.2 with protease inhibitor
92 tablets (cOmplete™, EDTA-free protease inhibitor cocktail, Merck, UK)) and sonicated 30 s on and 30
93 s off for three rounds using a XL-2020 sonicator (Heat Systems, USA). The sonicates were centrifuged
94 at 20 k x g for 30 min and the supernatant saved.

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95 **Acid precipitation:** the pH of the supernatant was reduced to pH 3.5 using HCl and stirred at room
96 temperature (RT) for 20 mins, then centrifuged at 60 k x g for 30 minutes. The pH of the supernatant
97 was then brought up to pH 7.5 with NaOH and stored overnight at 4 °C [11].

98 **$(NH_4)_2SO_4$ precipitation:** 47% w/vol of $(NH_4)_2SO_4$ was added to the supernatant[12] and stirred at RT
99 for 20 mins, then centrifuged at 60 k x g for 30 minutes. The pellet of protein was resuspended in 60
100 mL dialysis buffer (10 mM Tris, 1 mM EDTA pH 7.5) and dialysed overnight against the same buffer at
101 4 °C.

102 **Boiling:** For isolation of aSyn by boiling the *E. coli* pellet from 0.5 L of culture was resuspended in 50
103 mL high salt buffer (0.75 M NaCl, 100 mM MES, 1 mM EDTA pH 7)[13] and boiled in a water bath for 20
104 minutes at 100°C, then centrifuged at 60 k x g for 30 minutes. The supernatant was dialysed against
105 10 mM Tris 1 mM EDTA pH 7.5 overnight at 4 °C in SnakeSkin™ dialysis tubing, with a molecular
106 weight cut off (MWCO) of 10 kDa (Thermo Fisher Scientific, USA).

107 **Periplasmic lysis:** aSyn was released from the *E. coli* periplasm by osmotic lysis[2]. The pellet of *E. coli*
108 from 0.5 L of culture which had not been frozen was resuspended in 100 mL of osmotic shock buffer
109 (30 mM Tris, 40% sucrose (w/vol), 2 mM EDTA pH 7.2) and incubated at RT for 10 minutes. The
110 solution was centrifuged at 18 k x g for 20 minutes. The supernatant was discarded, and the pellet
111 resuspended in 90 mL of ice cold dH_2O with 37.5 µL of saturated $MgCl_2$ and kept on ice for 3 minutes
112 before centrifuging at 18 k x g for 20 minutes. The supernatant was dialysed overnight against 10
113 mM Tris, 1 mM EDTA pH 7.5 at 4 °C. The use of EDTA is particularly important after the addition of
114 $MgCl_2$ as it influences structure and aggregation rates[14].

115 **Ion exchange chromatography**

116 All buffers and aSyn samples for chromatography were filtered through a 0.22 µm filter and
117 degassed before use. For ion exchange chromatography (IEX) the protein was loaded onto a HiPrep
118 Q FF 16/10 anion exchange column (GE Healthcare, Sweden) and eluted against a linear gradient of 7
119 column volumes (CV) of IEX buffer B (10 mM Tris, 0.75 M NaCl, pH 7.5) followed by 2 CV of 100% IEX
120 buffer B using an ÄKTA Pure fast protein liquid chromatography (FPLC) system (GE Healthcare). To
121 determine the point of elution of aSyn from the chromatography column protein fractions which
122 were collected and monitored on absorption at 280 nm were ran on a 4-12% Bis-Tris gel (Invitrogen,
123 Thermo Fisher) using SDS-PAGE and stained with Coomassie blue. Fractions containing protein bands
124 corresponding to the predicted monomer aSyn molecular weight (MW) of 14.4 kDa were further
125 used in chromatographic steps. Fractions containing aSyn were pooled together and either dialysed
126 overnight in 20 mM Tris pH 7.2 and concentrated with a 10 K MWCO centrifugal concentrator to the

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127 desired concentration, ~130 – 140 µM and stored at -80 °C or were directly concentrated before gel
128 filtration.

129 **Hydrophobic interaction chromatography**

130 For aSyn isolated from the periplasm, we further optimised the purification protocol to yield a higher
131 purity of aSyn by the addition of a hydrophobic interaction chromatography (HIC) step. We changed
132 the counter ion from 0.75 M NaCl to 0.15 M $(NH_4)_2SO_4$ during IEX chromatography to remove a
133 dialysis step needed to exchange salts before subsequent HIC. The amount of $(NH_4)_2SO_4$ in the aSyn
134 solution post IEX was calculated based on the % of Buffer B it eluted at. Based on the volume of aSyn
135 protein collected, the amount of $(NH_4)_2SO_4$ needed to make the solution up to 1M was calculated
136 and then gradually added while stirring.

137 For HIC, the pH of the aSyn sample was adjusted to pH 7 and filtered through a 0.22 µm filter before
138 being loaded onto a HiPrep Phenyl FF 16/10 (High Sub) column (GE Healthcare, Sweden) and eluted
139 in HIC buffer A (50 mM Bis-Tris, 1 M $(NH_4)_2SO_4$ pH 7) against a linear gradient of 7 CV of HIC buffer B
140 (50 mM Bis-Tris pH 7) followed by 2 CV of 100% IEX buffer B. Fractions containing aSyn were pooled
141 and extensively dialysed against 20 mM Tris pH 7.2 overnight at 4 °C. The protein solution was
142 concentrated in 10 K MWCO centrifugal concentrators to the desired concentration, ~130 – 140 µM,
143 and frozen at -80 °C until further use.

144 **Gel filtration**

145 An aliquot of aSyn was defrosted and 500 µL of aSyn was injected into a gel filtration (GF) column,
146 Superdex 75 10/300 GL (GE Healthcare). The sample was eluted by isocratic elution at 0.8 mL/min in
147 20 mM Tris pH 7.2. Tubing between the injection point and the fraction collector on the ÄKTA Pure
148 FPLC system was changed from orange (0.5 mm) to blue (0.25 mm) to reduce dilution of the protein
149 sample and give a narrower collection peak. Monomeric aSyn eluted at ~ 9 mL.

150 **Densitometry to determine purity of aSyn**

151 Fractions of proteins samples were run on 4-12% Bis-Tris gels using SDS-PAGE for separation of
152 proteins based on size. The gels were stained with Coomassie blue and the gel image analysed using
153 ImageJ software[15] to determine the percentage of aSyn present. Regions of interest were selected
154 and a histogram of the intensity of dyed protein in the area displayed. From the histogram the area
155 of aSyn was calculated as a percentage of the total area of stained proteins to give the percentage
156 purity.

157 **Reverse phase high pressure liquid chromatography to determine the purity of aSyn**

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158 The purity of the aSyn samples was analysed by analytical reversed phase chromatography (aRP) on
159 a 1260 Infinity high pressure liquid chromatography (HPLC) system (Agilent Technologies LDA UK
160 Limited, UK), equipped with an autosampler and a diode-array detector. 50 µL of sample was
161 injected onto a Discovery BIO Wide Pore C18 column (15 cm x 4.6 mm, 5 µm column with a guard
162 column) (Supelco, Merck, UK) and eluted on a gradient of 95% water + 0.1% acetic acid and 5%
163 acetonitrile + 0.1% acetic acid to 5% water + 0.1% acetic acid and 95% acetonitrile + 0.1% acetic acid
164 at a 0.8 mL/min flow-rate over 40 mins. The elution profile was monitored by UV absorption at 220
165 and 280 nm. The area under the peaks in the chromatograph of absorption at 280 nm was calculated
166 to provide the percentage purity of aSyn. aSyn eluted at ~17.9 mins.

167 **Native mass spectrometry**

168 Non–denaturing nano-electrospray ionization mass spectrometry (Native mass spectrometry) was
169 used to analyse the oligomerisation states of recombinant aSyn prepared in four different ways:
170 boiled, $(NH_4)_2SO_4$ ppt, acid ppt and periplasmic lysis. Native mass spectra were recorded on a Synapt
171 HD mass spectrometer (Waters, Manchester, UK) modified for studying high masses. Protein
172 samples were exchanged into 0.20 M ammonium acetate (pH 7.0) solution using Micro Bio–Spin 6
173 chromatography columns (Bio– Rad, USA) and diluted to a final concentration of 5–10 µM before
174 analysis. An aliquot of 2.5 µL of protein solution was electrosprayed from a borosilicate emitter
175 (Thermo Scientific, UK) for sampling. Typical conditions for the data acquire were capillary voltage
176 1.6-2.2 kV, cone voltage 160– 190 V, Trap 40−50 V, Transfer 140 V with backing pressure 3–4 mbar
177 and source temperature of 20 °C. Spectra were calibrated externally using caesium iodide. Data
178 acquisition and processing were performed using MassLynx 4.1. Spectra were edited manually using
179 Adobe Illustrator for the purpose of this publication.

180 **Thioflavin-T based kinetic aggregation assays**

181 20 µM freshly made thioflavin-T (ThT) (abcam, Cambridge, UK) was added to 50 µL of 20 µM aSyn
182 after GF in 140 mM KCl, 20 mM Tris pH 7.2. All samples were loaded onto nonbinding, clear bottom,
183 96-well half-area plates (Greiner Bio-One GmbH, Germany). The plates were sealed with a
184 SILVERseal aluminium microplate sealer (Grenier Bio-One GmbH). Fluorescence measurements were
185 taken using a FLUOstar Omega plate reader (BMG LABTECH GmbH, Ortenbery, Germany). The plates
186 were incubated at 37°C with double orbital shaking at 300 rpm for five minutes before each read
187 every hour for 170 hours. Excitation was set at 440 nm with 20 flashes and the ThT fluorescence
188 intensity measured at 480 nm emission with a 1300 gain setting. ThT assays were repeated twice
189 using four wells for each condition. For aSyn isolated by boiling, four gel filtrated samples were used,
190 for aSyn isolated by $(NH_4)_2SO_4$ ppt three gel filtrated samples were used, for aSyn isolated by acid

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191 ppt two gel filtrated samples were used, and for aSyn isolated by periplasmic lysis four gel filtrated
192 sampled were used. Data were normalised to the sample with the maximum fluorescence intensity
193 for each plate.

194 **Analytical size exclusion chromatography to determine the remaining aSyn monomer**
195 **concentration after aggregation assays**

196 At the end of the ThT-based aggregation assays, the amount of remaining monomer of aSyn in each
197 well was determined by analytical size exclusion chromatography on a HPLC (SEC-HPLC). The
198 contents of each well after the ThT-based assay were centrifuged at 21k x g for 20 minutes and the
199 supernatant was added to individual aliquots in the autosampler of the Agilent 1260 Infinity HPLC
200 system (Agilent Technologies LDA UK Limited, UK). 25 µL of each sample was injected onto an
201 Advance Bio SEC column, 7.8 x 300 mm 300Å (Agilent, UK) in 20 mM Tris pH 7.2 at 1 mL/min
202 flowrate. Injections were also made for each sample at the start of the assay to quantify the amount
203 of starting protein. The elution profile was monitored by UV absorption at 220 and 280 nm. The
204 remaining monomer percentage was calculated from the ratio of the area under the curves at the
205 beginning and the end of the assay.

206 **Transmission Electron Microscopy**

207 20 µL of aSyn was taken directly from the ThT-based aggregation assay plates of the boiled,
208 $(NH_4)_2SO_4$ ppt and periplasmic lysis samples and diluted 1:4 with dH_2O. The acid ppt aSyn sample was
209 used neat, and all samples were incubated on glow-discharged carbon coated copper grids for 1 min
210 before washing twice with dH_2O. 2% uranyl acetate was used to negatively stain the samples for 30 s
211 before imaging on the Tecnai G2 80-200kv transmission electron microscopy (TEM) at the Cambridge
212 Advanced Imaging Centre.

213

214 **Results**

215 **Acid precipitation of *E. coli* proteins leads to the highest purity of aSyn before chromatography**

216 Four commonly used protocols for the purification of recombinant aSyn were compared to
217 determine which yielded the highest proportion of monomeric aSyn and the highest degree of
218 purity. First, in all four protocols, 0.5 L of *E. coli* culture was induced for four hours with IPTG before
219 pelleting the bacteria. The pellets were then treated differently dependent on the isolation protocol.
220 For boiled samples, the *E. coli* pellet was resuspended in a high salt buffer then boiled in a water
221 bath at 100 °C for 20 minutes before centrifuging. The supernatant was dialysed overnight in 10 mM

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222 Tris, 1 mM EDTA pH 7.5. For the acid and $(NH_4)_2SO_4$ ppt protocols, the *E. coli* pellets were
223 resuspended in 10 mM Tris, 1 mM EDTA pH 7.5 including protease inhibitors before being sonicated
224 and centrifuged. The supernatant was then precipitated either by reducing the pH to 3.5 with HCl on
225 a stirrer for 20 mins or by addition of 47% (w/vol) $(NH_4)_2SO_4$ on a stirrer for 20 mins. The precipitates
226 were centrifuged, the supernatant from the acid ppt was brought back to neutral pH with NaOH and
227 stored overnight at 4 °C before chromatography was performed. The pellet from the $(NH_4)_2SO_4$ ppt
228 containing the aSyn was resuspended in 10 mM Tris, 1 mM EDTA pH 7.5 and dialysed overnight. For
229 periplasmic lysis, the *E. coli* periplasm was lysed by osmotic shock. The *E. coli* pellet was resuspended
230 in a sucrose based buffer which acts as an osmotic stabiliser preventing whole cell lysis[16]. After
231 centrifugation to pellet the *E. coli,* the outer membrane was lysed by osmotic shock with water and
232 $MgCl_2$ to release the contents of the periplasm, but not the cytoplasm. The lysed protein was
233 dialysed overnight in 10 mM Tris, 1 mM EDTA pH 7.5. The acid precipitated aSyn sample was the
234 most pure at this point, with 96.5% purity by densitometry measurement of the SDS-PAGE
235 Coomassie stained gel, which agrees with a previous study[7] (Supplementary Figure 1, Supplementary
236 Table 1).

237 **Chromatographic isolation of aSyn yields 80-95% pure aSyn**

238 IEX was then used to isolate aSyn from all protein solutions using a HiPrep™ Q FF 16/10 anion
239 exchange column. aSyn was eluted on a linear gradient of IEX buffer A (10 mM Tris, 1 mM EDTA pH
240 7.5) against IEX buffer B (10 mM Tris, 1 mM EDTA, 0.75 M NaCl, pH 7.5) (Figure 1 a.i, b.i, c.i, d.i). To
241 determine which fractions the aSyn resided in, the samples were analysed by SDS-PAGE and the gel
242 stained by Coomassie blue to visualise the protein. Fractions containing aSyn are highlighted in the
243 coloured block on the IEX chromatograms (Figure 1 a.i,ii, b.i,ii, c.i,ii, d.i,ii). The purity of the samples
244 was analysed by densitometry and the aSyn precipitated in acid and the aSyn that was boiled were
245 found to be 100% and 99.3% pure, respectively (Supplementary Table 1). After IEX the aSyn samples
246 were dialysed in 20 mM Tris pH 7.2 and concentrated using centrifugal concentrators with a MWCO
247 of 10 kDa. aSyn was concentrated until the protein concentration was between 130-140 μM before
248 storage at -80°C. To increase the purity of the aSyn from samples isolated by periplasmic lysis and
249 $(NH_4)_2SO_4$ ppt further, and to ensure isolation of monomeric protein, gel filtration was used. 500 μL
250 of aSyn was injected onto a Superdex 75 10/300 GL column and eluted isocratically (Figure 1, a.iii,
251 b.iii, c.iii, d.iii.) The Coomassie blue stained gel after SDS-PAGE of aSyn showed that the all isolation
252 protocols, apart from periplasmic lysis (Figure 1d.iv), lead to 100% pure aSyn after IEX and GF (Figure
253 2a., Supplementary Table 1). The aSyn sample isolated by periplasmic lysis still contained
254 contaminating proteins, aSyn was only 91.7% pure in fraction 1 and 97.7% pure in fraction 2 (Figure
255 1div stars indicate the contaminants, Supplementary Table 1). Previous protocols using the

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256 periplasmic lysis protocol have also employed an extra hydrophobic interaction chromatography
257 (HIC) step[17]. An additional HIC step was added using a HiPrep™ Phenyl Fast Flow (high sub) 16/10
258 column, but the previous protocol was updated to save time by substituting the counter ion salt in
259 IEX from NaCl to $(NH_4)_2SO_4$ to prevent an additional buffer exchange step before HIC. Therefore,
260 directly after IEX $(NH_4)_2SO_4$ was added to make the protein solution up to 1 M $(NH_4)_2SO_4$, equivalent
261 to the starting buffer A for HIC. aSyn was eluted on a linear gradient against HIC buffer B (50 mM Bis
262 Tris, pH 7) (Figure 1d.v, d.vi). aSyn was 100% pure when analysed by densitometry after IEX, HIC and
263 GF (Figure 2a., Supplementary Table 1). Supplementary Figure 2 and Supplementary Table 2 shows a
264 second purification run for each purification method and the concentration of protein in each step
265 of purification, showing the methods to be reproducible.

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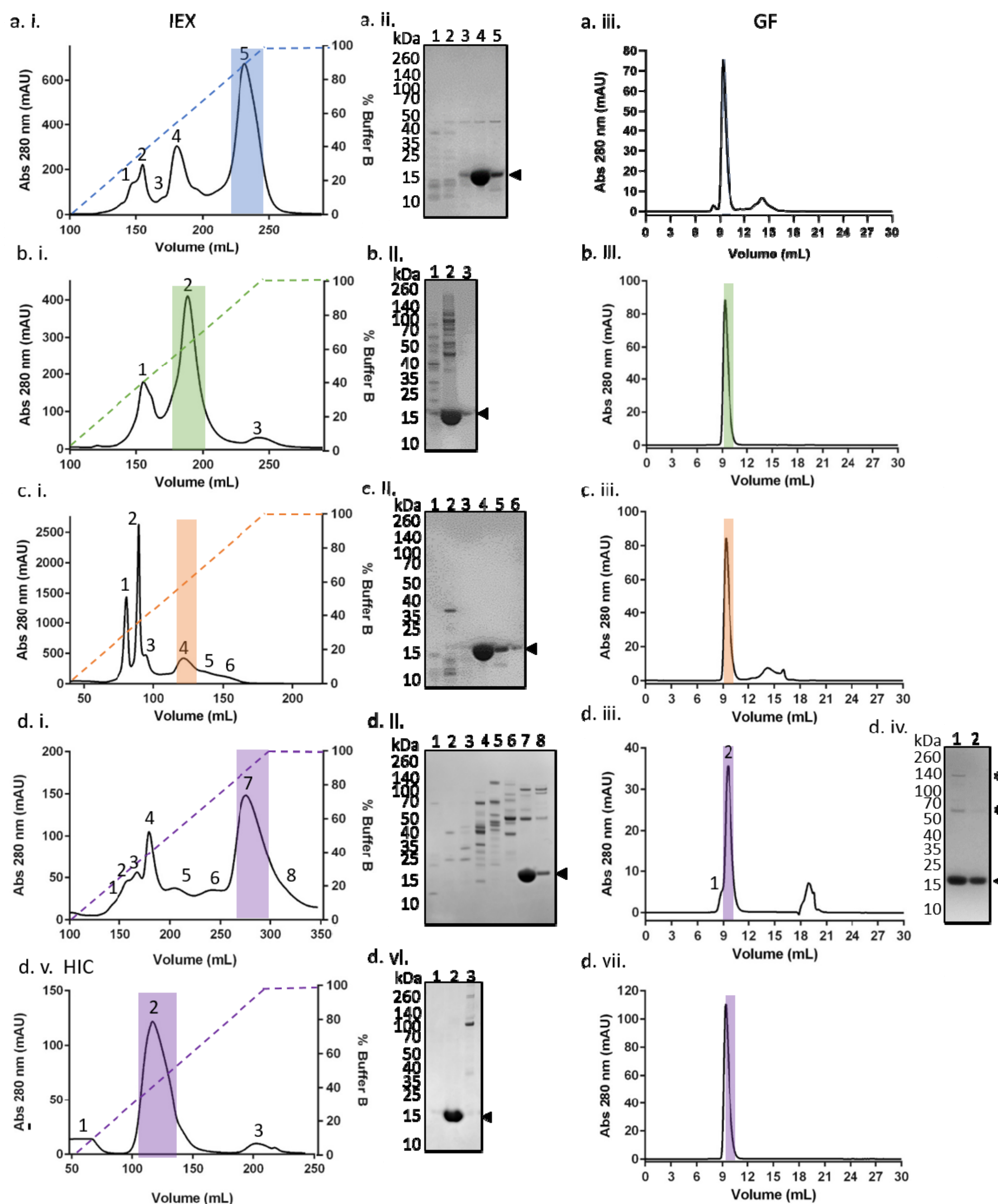

266

267 **Figure 1. aSyn isolated by boiling, acid ppt and $(NH_4)_2SO_4$ ppt is highly pure after IEX and GF, while**
268 **aSyn isolated by periplasmic lysis requires an additional HIC step to increase purity.** aSyn was
269 isolated by IEX from samples that were (a.i.) boiled, (b.i.) precipitated by $(NH_4)_2SO_4$, (c.i.) precipitated
270 by acidification and (d.i.) lysed from the periplasm. Protein fractions from IEX were taken from
271 individual peaks with maximum absorption at 280 nm and analysed by SDS-PAGE using a 4-12% bis-
272 tris gel which was stained by Coomassie blue, (a.ii.) boiled, (b.ii.) precipitated by $(NH_4)_2SO_4$, (c.ii.)
273 precipitated by acidification and (d.ii.) lysed from the periplasm. aSyn ran at ~ 15 kDa, indicated by

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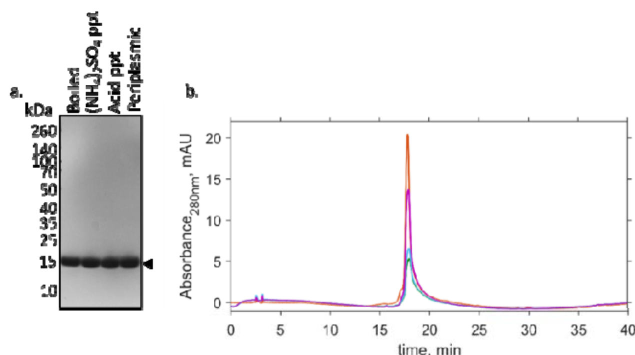
274 the arrow next to the gel images, and the peak aSyn resided in is highlighted in colour on the IEX
275 chromatographs. GF of pooled fractions containing aSyn after IEX show monomeric aSyn eluting
276 after ~9 mL, (a.iii.) boiled, (b.iii.) precipitated by $(NH_4)_2SO_4$, (c.iii.) precipitated by acidification and
277 (d.iii.) periplasmic lysis. GF of aSyn isolated by periplasmic lysis did not yield as highly pure aSyn as
278 the other methods did, (d.iv.) as indicated by the presence of contaminating proteins (*) in the
279 Coomassie blue stained gel. Therefore, an additional (d.v.) HIC step was added and (d.vi.) aSyn was
280 shown to be subsequently purer as shown by the Coomassie blue stained gel. (d.vii.) The final GF of
281 aSyn isolated by periplasmic lysis after IEX and HIC showed a single peak of monomeric aSyn eluting
282 at ~ 9 mL.

283 Densitometry analysis of the Coomassie blue stained gel of the four aSyn samples after GF showed
284 100% pure monomeric aSyn in all samples. However, analytical reversed phase chromatography
285 (aRP) was also employed to determine the purity of each sample as it is a more sensitive method to
286 detect contaminants (Figure 2b). The samples were shown to be less pure after IEX by aRP compared
287 to densitometry measurements (Supplementary Figure 3a), sample purity ranged from 62.9 % to
288 84.8% when analysed by aRP, but ranged between 49.7 and 100% pure when analysed by
289 densitometry (Table 1 and Supplementary Table 1). aRP of aSyn purified by IEX, HIC and GF
290 compared to only IEX and GF led to an increase in purity from 63.5% to 95% (Supplementary Figure
291 3b). After GF the aSyn purity was determined to be 86% for the boiled sample, 81% for $(NH_4)_2SO_4$
292 ppt, 89.9% for acid ppt and 95% for periplasmic lysis of aSyn by aRP (Figure 2b, Table 1).



293

294 **Figure 2. Coomassie blue stained gel and reverse phase chromatographs of aSyn after gel filtration**
295 **show highly pure monomeric aSyn.** (a.) Samples of aSyn after gel filtration were analysed by SDS-
296 PAGE on a 4-12% Bis-Tris gel and stained with Coomassie blue. aSyn appears as a single band,
297 indicated by the arrow at around 15 kDa. (b.) 50 µL of each sample was injected onto an analytical
298 Discovery BIO Wide Pore C18 column to determine the purity of aSyn. aSyn isolated by boiling (blue)
299 was 86% pure, aSyn isolated by $(NH_4)_2SO_4$ ppt (green) was 81% pure, aSyn isolated by acid ppt was

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300 (orange) 89.9% pure and aSyn isolated from periplasmic lysis (purple) was 95% pure, determined by
301 the area under the peak.

302 **Table 1. Purity of aSyn at different steps of isolation determined by reverse phase chromatography**

	Boiled			$(NH_4)_2SO_4$ ppt			Acid ppt			Periplasmic lysis		
	Total protein (mg/mL)	% purity	Final aSyn conc. (μM)	Total protein (mg/mL)	% purity	Final aSyn conc. (μM)	Total protein (mg/mL)	% purity	Final aSyn conc. (μM)	Total protein (mg/mL)	% purity	Final aSyn conc. (μM)
Post IEX	1.038	69.3		0.619	84.8		1.59	62.9		0.458	82.5	
Post HIC	-	-		-	-		-	-		0.254	86.7	
GF	0.219	86	36.9	0.267	81	44.7	0.257	89.9	42.9	0.292	95	48.7

303

304 **Analysis of aSyn samples by native mass spectrometry shows that the acid precipitation protocol**
305 **yields the most highly monomeric aSyn**

306 Obtaining highly pure monomeric aSyn is needed for the majority of assays performed. Although the
307 SDS-PAGE Coomassie blue stained gel and aRP methods show monomeric aSyn, the SDS used in the
308 PAGE and the organic solvents used in aRP are denaturing and may give a false impression of the
309 level of monomeric protein present. Instead, we employed non-denaturing nano electrospray
310 ionization mass spectrometry (native MS). The technique permits the study of protein structure at
311 physiological pH and the identification of aSyn multimers and degradation products without the
312 need to use cross-linkers which may alter structure or induce artefacts[18]. In the boiled sample, aSyn
313 was found in both monomer and dimer form, but also as a degraded product of 11562±3 Da
314 comprising 36.6% of the sample (Figure 3a, Table 2). A degraded product of 12172 Da was also
315 identified by Giehm, et al., after boiling of aSyn[6]. The percentage of aSyn products were calculated
316 by the relative intensity of the m/z peaks in each charge state (Supplementary Table 3). As the
317 degraded product was not detected by SDS-PAGE or aRP it may have been induced during
318 electrospray ionisation. aSyn samples precipitated in $(NH_4)_2SO_4$ contained monomer (90.3%), dimer
319 (8.5%) and trimer (1.2%) (Figure 3b, Table 2), while the aSyn isolated by acid ppt contained only
320 monomeric aSyn (Figure 3c, Table 2). aSyn isolated by periplasmic lysis was highly monomeric
321 (96.5%) with a small percentage of dimer (3.5%) (Figure 3d, Table 2). The monomer was disordered
322 in all samples, as expected, and the dimer and trimers were possibly linked by non-covalent bonds
323 which remain formed during non-denaturing electrospray ionisation.

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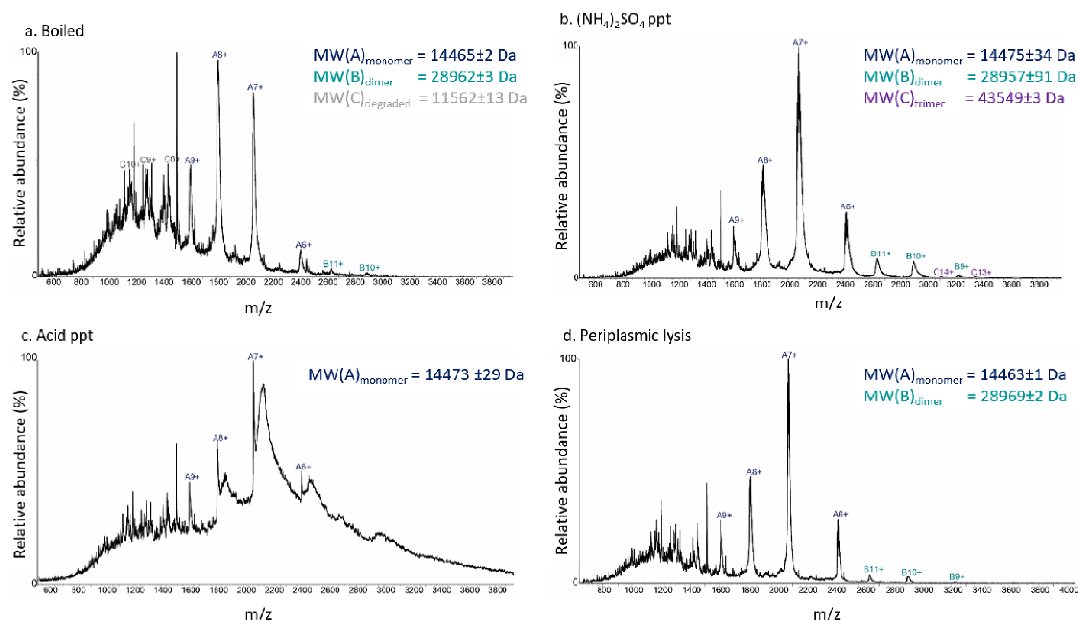

Figure 3. Native MS data of aSyn after gel filtration shows that acid precipitation yields the highest percentage of monomeric aSyn. aSyn in 200 mM $NH_4CH_3CO_2$ was analysed by native MS. aSyn isolated by (a.) boiling was found as a monomer (A) in charge states 9+ to 6+, the MW is highlighted in blue, as a dimer (B), highlighted in teal, in charge states 10+, 11+ and as a potentially degraded product (C) highlighted in grey at charge states 10+ to 8+ with a MW of ~11562 Da. aSyn isolated with (b.) $(NH_4)_2SO_4$ ppt was identified as monomeric (A), dimeric (B) and trimeric (C) (~43549 Da) protein forms, the trimer charge states 14+, 13+ and MW are highlighted in purple. The aSyn sample isolated with (c.) acid ppt was only found in a monomeric state (A), while aSyn isolated by (d.) periplasmic lysis was found to be in monomeric (A) and dimeric (B) forms.

Table 2. Molecular weight and relative percentage of aSyn structures in each sample determined by native nano-ESI-MS

	Boiled		**$(NH_4)_2SO_4$ ppt**		**Acid ppt**		**Periplasmic lysis**	
	MW	%	MW	%	MW	%	MW	%
Degraded	11562 ± 13	36.6	-	-	-	-	-	-
Monomer	14465.4 ± 2.2	60.2	14475 ± 34	90.3	14473 ± 29	100	14463 ± 1	96.5
Dimer	28962.5 ± 3.0	3.2	28957 ± 91	8.5	-	-	28969 ± 2	3.5
Trimer	-	-	43549 ± 3	1.2	-	-	-	-

To determine whether the difference in percentage of monomeric, dimeric and trimeric aSyn affects the aggregation rate, we performed kinetic aggregation assays using the molecule ThT which fluoresces when bound to fibrillated forms of aSyn, providing a kinetic readout[19]. 20 µM of aSyn was

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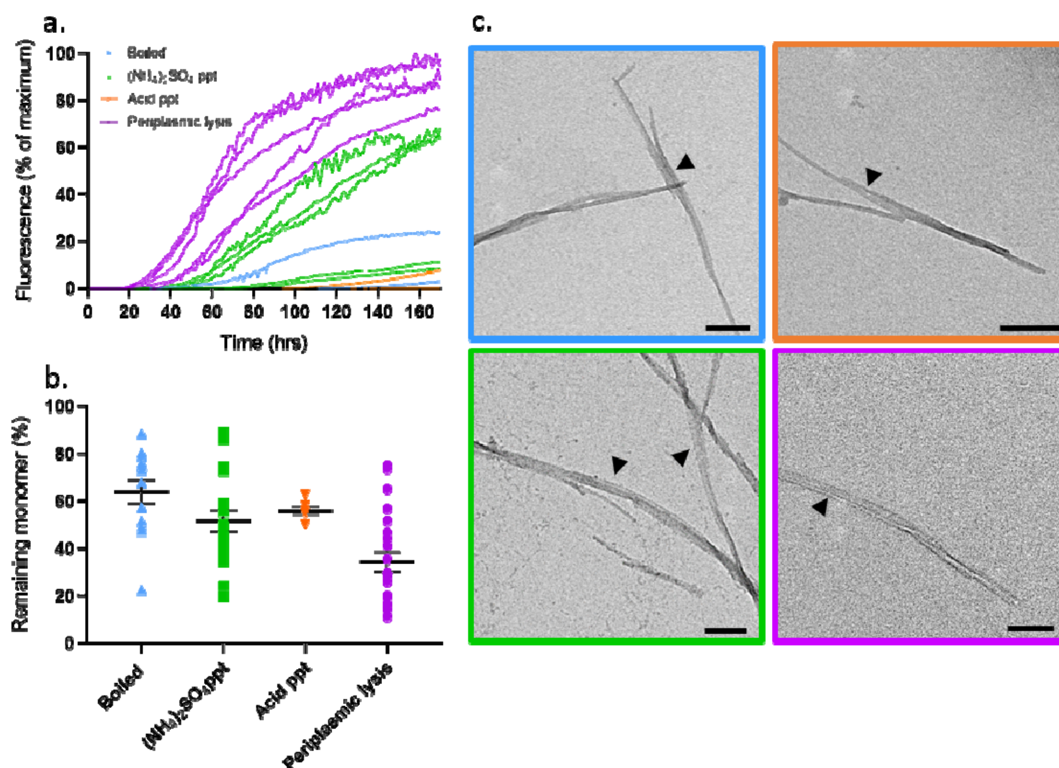
341 incubated with 20 µM of ThT in 20 mM Tris, 100 mM KCl pH 7.2 for one week. The kinetic
342 aggregation curves show that the aSyn isolated by periplasmic lysis was the most aggregation prone,
343 followed by aSyn isolated by $(NH_4)_2SO_4$ ppt (Figure 4a). Surprisingly, aSyn isolated by boiling and acid
344 ppt appeared to be the least aggregation prone under the conditions tested (Figure 4a). As it is
345 known that ThT assays are highly variable and that ThT also has varying fluorescence intensities
346 when bound to different fibril polymorphs[19,20], we also performed analytical size exclusion
347 chromatography on a HPLC (SEC-HPLC) to determine the quantity of remaining aSyn monomer. We
348 observe that the quantity of the remaining aSyn monomer does not fully reflect the ThT
349 fluorescence observed, where at least 40% of the acid ppt aSyn appears to have formed higher order
350 structures (Figure 4b). However, the remaining monomer concentration does reflect the trend
351 observed in the ThT-based assays, whereby the aSyn isolated by periplasmic lysis had the least
352 remaining monomer and aSyn isolated by $(NH_4)_2SO_4$ ppt also had less remaining monomer than the
353 samples that were boiled and precipitated by acid. We further investigated the morphology of the
354 aSyn samples to determine whether fibrils had formed and whether their morphology differed using
355 TEM. TEM showed fibrils present in all samples, but fibrils were harder to find in the sample from
356 acid ppt, indicating less fibrils were present. All fibrils have straight morphology (Figure 4c,
357 Supplementary Figure 4), as shown previously for aSyn aggregated in the presence of salt[21]. The fibril
358 bundles also showed lateral binding (Figure 4c, shown by the arrows).



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360 **Figure 4. Kinetic aggregation assays show aSyn isolated by periplasmic lysis is the most**
361 **aggregation prone and all aSyn samples have straight fibril morphology.** (a.) ThT-based aggregation
362 assays show aSyn isolated by periplasmic lysis (purple, n=5) and $(NH_4)_2SO_4$ ppt (green, n=5)
363 aggregate at a faster rate and to a greater extent than aSyn isolated by boiling (blue, n=4) or acid ppt
364 (orange, n=3). Each sample (n) represents aSyn from individual GF runs plated as four well replicates
365 over two plates. 20 µM aSyn in 20 mM Tris, 100 mM KCl pH 7.2 was incubated with 20 µM ThT in a
366 half area 96 well plate with double orbital agitation at 300 rpm for 5 minutes before each read every
367 hour for 170 hours. (b.) The percentage of the remaining monomer concentration in each well after
368 the ThT assay was determined by performing SEC-HPLC and calculating the area under the curve
369 compared to the area under the curve of the starting monomeric sample. Error bars represent SEM
370 from wells n=14 boiled, n=19 $(NH_4)_2SO_4$, n=7 acid, n=22 periplasmic. (c.) aSyn samples were taken
371 directly from the ThT wells, boiled (blue), $(NH_4)_2SO_4$ ppt (green) and periplasmic lysis (purple)
372 samples were diluted 1:4 and the acid precipitated (orange) sample was used neat when incubating
373 on grids before being imaged by TEM. All samples showed fibrils with straight morphology and
374 lateral binding between fibril bundles (black arrows). Scale bar = 200 nm.

375

376

377 **Discussion**

378 Many different protocols are currently used for the purification of aSyn, yet little investigation has
379 been performed into the exact product that is present at the end of these purification methods.
380 Here, we compared four commonly applied protocols, boiling, acid ppt, $(NH_4)_2SO_4$ ppt and
381 periplasmic lysis to determine which of the four methods yielded the highest protein purity and the
382 most monomeric aSyn. Isolation of aSyn by acid ppt and periplasmic lysis yielded the highest
383 percentage of monomeric protein, at 100% and 96.5% respectively, and 89.9% and 95% purity,
384 respectively. The aggregation rate of aSyn prepared by these two methods varied greatly when
385 monitored using a ThT-based kinetic assay, where aSyn isolated by acid ppt barely aggregated, yet
386 aSyn isolated by periplasmic lysis aggregated well under the conditions used. The remaining
387 monomer concentration did not reflect the amount of fibrillisation of aSyn isolated by acid ppt,
388 possibly indicating that more oligomeric structures or amorphous aggregates had formed which
389 were not detected by ThT fluorescence or SEC-HPLC. This may indicate that the method of aSyn
390 isolation can impact the aggregation propensity of the dynamic ensemble of monomer
391 conformations.

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392 Further work is needed to determine whether the purification protocols we use can influence the
393 conformation dynamics of aSyn. Currently techniques are not sensitive enough to determine if we
394 are skewing the dynamic equilibrium of conformations by using different purification techniques,
395 which could then impact the propensity of aSyn to aggregate, or even the subsequent fibril
396 polymorphs formed and its toxicity[22]. It is thus important to characterise the sample fully to
397 guarantee reproducibility and validity of the data.

398

399 **Author Information**

400 **Corresponding Author**

401 Email: asds2@cam.ac.uk, gsk20@cam.ac.uk

402 **Author Contributions**

403 A.D.S Designed experiments and performed purification, kinetic assays and TEM. D.M-V. performed
404 native MS. A.D.S and G.S.K wrote the manuscript. All authors have given final approval of the
405 manuscript.

406 **Notes**

407 The authors declare no competing financial interests.

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411 loading into the TEM. This work was supported by Wellcome Trust, Alzheimer's Research UK Grants,
412 the Michael J Fox Foundation and Infinitus China Ltd.

413 **Supplementary Information**

414 Raw data is available at the University of Cambridge Data Repository. Supplementary Information
415 contains; **Supplementary Figure 1.** Coomassie blue stained 4-12% Bis-Tris gel of aSyn samples
416 isolated by different methods shows acid precipitation yields the highest purity of aSyn prior to
417 chromatography. **Supplementary Table 1.** Purity of aSyn determined by densitometry of Coomassie
418 blue stained gels. **Supplementary Figure 2.** The second purification run also shows that unlike the
419 other isolation protocols aSyn isolated by periplasmic lysis requires an additional HIC step to
420 increase purity. **Supplementary Table 2.** Concentration of total protein and aSyn during purification
421 run one and two. **Supplementary Table 3.** Peak intensity of degraded product, monomer, dimer and

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422 trimers from native MS used to calculate the percentage of aSyn structures in each sample.

423 **Supplementary Figure 3.** Analytical reverse phase chromatography shows more impurities than

424 Coomassie blue stained gels. **Supplementary Figure 4.** Straight fibrils are formed during ThT assays

425 from all purification methods used. **Supplementary Figure 5**. All raw images of Coomassie blue

426 stained SDS-PAGE gels used in this publication.

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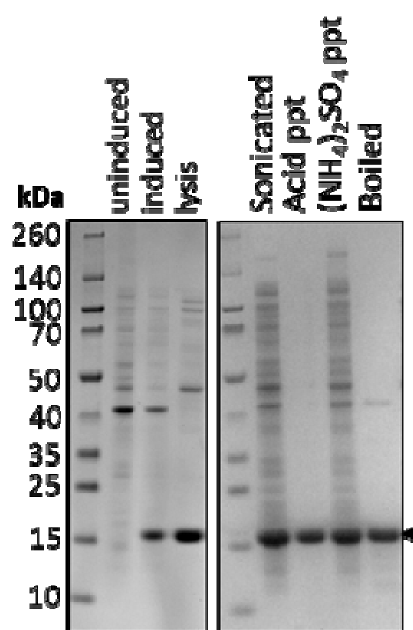
522 **Supplementary Information**



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524 **Supplementary Figure 1. Coomassie blue stained 4-12% Bis-Tris gel of aSyn samples**
525 **isolated by different methods shows acid precipitation yields the highest purity of aSyn**
526 **prior to chromatography.** Samples of E. coli before (uninduced) and after induction
527 (induced) with 1 mM IPTG show expression of aSyn ~15 kDa shown by the black arrow in the
528 induced lane. aSyn was then extracted from E. coli by different methods: aSyn lysed from the
529 periplasm after osmotic shock is shown as periplasmic lysate (lysis). Prior to precipitation
530 methods, aSyn was sonicated to break open the E. coli (sonicated) before i) acid
531 precipitation and recovery of the supernatant containing aSyn by centrifugation (Acid ppt),
532 ii) precipitation with $(NH_4)_2SO_4$ and iii) recovery of aSyn from the pellet after centrifugation
533 ($(NH_4)_2SO_4$ ppt). E. coli were also boiled and the aSyn recovered in the supernatant after
534 centrifuging (Boiled).
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545 **Supplementary Table 1. Purity of aSyn determined by densitometry of Coomassie blue stained gels**
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Isolation protocol	aSyn % purity		
	PreIEX	Post IEX	Post GF
Boiled	91.1	99.3	100.0
$(NH_4)_2SO_4$ ppt	37.6	49.7	100.0
Acid ppt	96.5	100.0	100.0
Periplasmic Lysis	70.0	92.2	Fr1 91.7 Fr2 97.7

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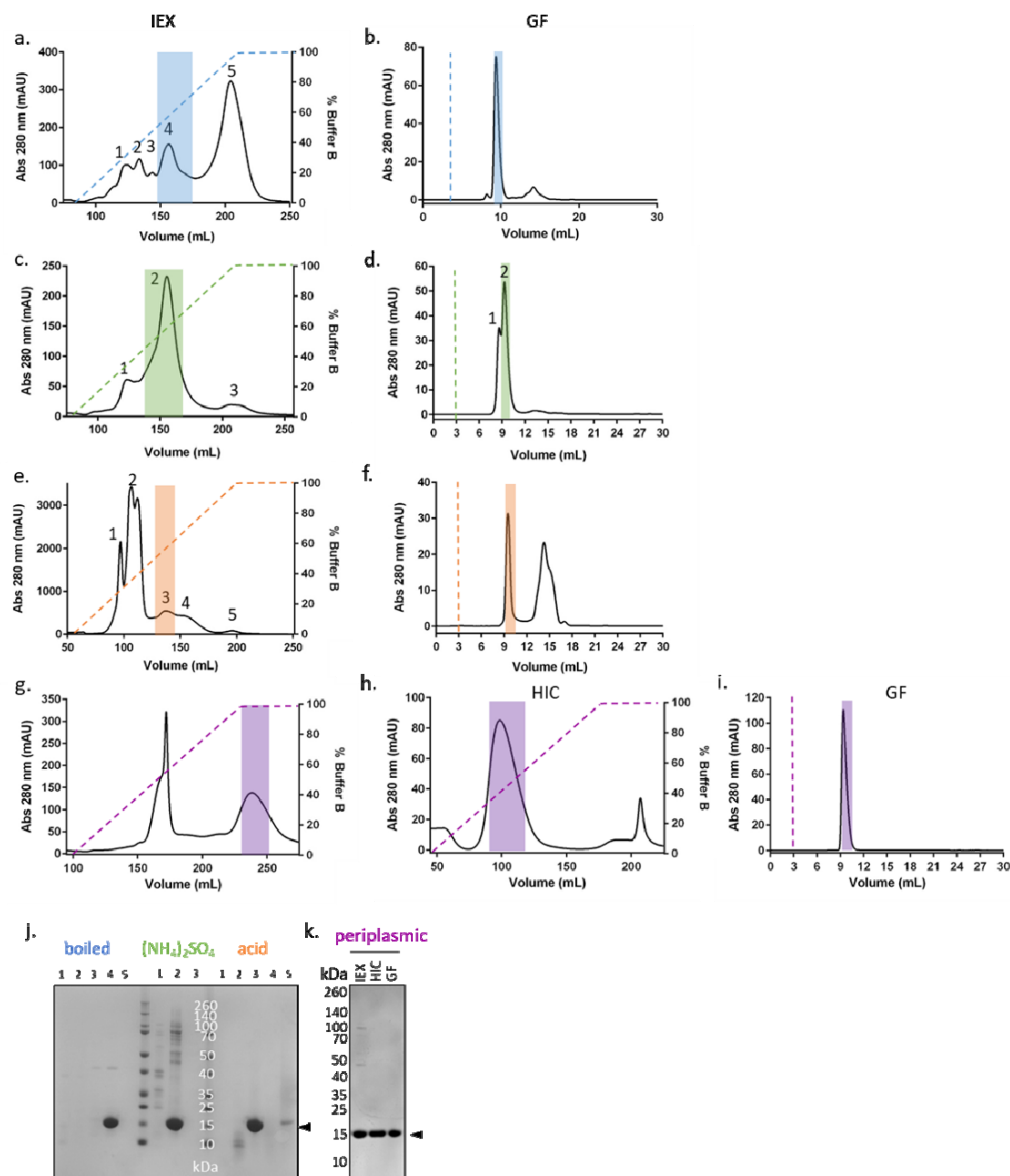

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573 **Supplementary Figure 2. The second purification run also shows that unlike the other**
574 **isolation protocols aSyn isolated by periplasmic lysis requires an additional HIC step to**
575 **increase purity.** Chromatograph of IEX chromatography of aSyn isolated by (a.) boiling E. coli,
576 (c.) $(NH_4)_2SO_4$ precipitation, (e.) acid precipitation and (g.) periplasmic lysis, followed by GF
577 for the (b.) boiled aSyn, (d.) $(NH_4)_2SO_4$ precipitation and (f.) acid precipitation. aSyn from
578 periplasmic lysis was further purified by (h.) hydrophobic interaction chromatography (HIC)
579 before (i.) GF. A Coomassie blue stained gel of fractions separated by SDS-PAGE from the
580 eluted protein peaks of the IEX chromatography were used to determine which peak aSyn
581 resided in (j), shown by the black arrow ~ 15 kDa, and correspond to the highlighted regions
582 in the IEX graphs (a.,c.,e.). (k) Protein fractions from IEX, HIC and GF for aSyn from
583 periplasmic lysis show aSyn by the black arrow and correspond to the highlighted areas in g.,
584 h., i.,.

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586 **Supplementary Table 2. Concentration of total protein and aSyn during purification run**
587 **one and two**

Purification state	Isolation method	mg/mL	uM aSyn
1. PreIEX	Acid ppt	4.2	
	Boiled	1.28	
	NH42SO4 ppt	0.991	
	Periplasmic lysis	3.37	
1. Post IEX	Acid ppt	2.275	
	Boiled	0.889	
	NH42SO4 ppt	0.683	
	Periplasmic lysis	0.75	
1. Post HIC	Periplasmic lysis	0.55	
1. Post GF	Acid ppt	0.251	42.11
	Boiled	0.158	26.51
	NH42SO4 ppt	0.136	22.82
	Periplasmic lysis	0.9	65.00
2. Post IEX	Acid ppt	1.590	
	Boiled	1.038	
	NH42SO4 ppt	0.620	
	Periplasmic lysis	0.458	
2. Post HIC	Periplasmic lysis	0.254	
2. Post GF	Acid ppt	0.257	42.95
	Boiled	0.219	36.75
	NH42SO4 ppt	0.267	44.78
	Periplasmic lysis	0.292	48.90

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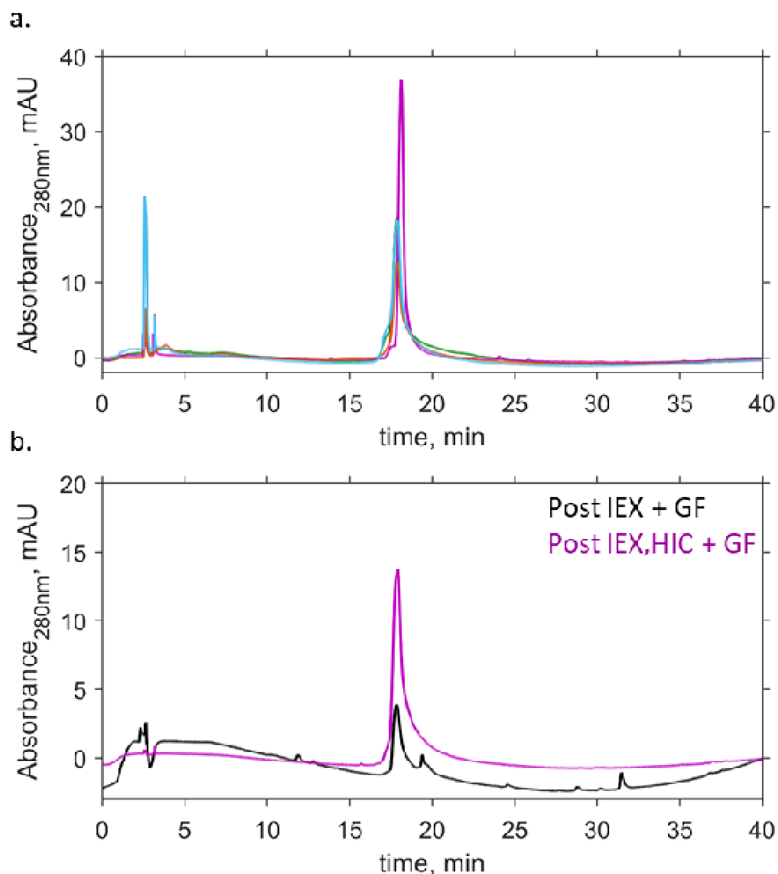

604

605 **Supplementary Figure 3. Analytical reverse phase chromatography shows more impurities**
606 **than Coomassie blue stained gels.** Pooled fractions of aSyn after IEX were analysed by
607 analytical reverse phase chromatography (aRP) to determine purity. 50 µL of sample was
608 injected into a C18 column and aSyn was eluted on a gradient of 95% water with 0.1% acetic
609 acid and 5% acetonitrile with 0.1% acetic acid at 0.8 mL/min. (a) The purity of aSyn isolated
610 by boiling (blue), acid precipitation (orange), $(NH_4)_2SO_4$ precipitation (green) and periplasmic
611 lysis (purple) was determined from the area under the peaks, aSyn eluted ~ 17.8 mins. (b)
612 Purity of aSyn after IEX and GF (black) compared to IEX, HIC and GF (purple) shows fewer
613 contaminating proteins in the sample which had the additional HIC step.

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Supplementary Table 3. Peak intensity of degraded protein, monomer, dimer and trimers from native MS used to calculate the percentage of aSyn structures in each sample

	degradation product charge states			monomer charge states				dimer charge states				trimer charge states			
Sample	C10+	C9+	C8+	A9+	A8+	A7+	A6+	B11+	B10+	B9+	B8+	C14+	C13+	C12+	Total m/z intensity
Boiled	53	53	55	54	106	90	13	8	4	2	0	0	0	0	437
$(NH_4)_2SO_4$ ppt	0	0	0	159	338	692	199	61	52	12	6	6	7	5	1537
Acid ppt	0	0	0	116	182	93	42	0	0	0	0	0	0	0	2832
Periplasmic lysis	0	0	0	112	189	397	113	15	12	2	0	0	0	0	840

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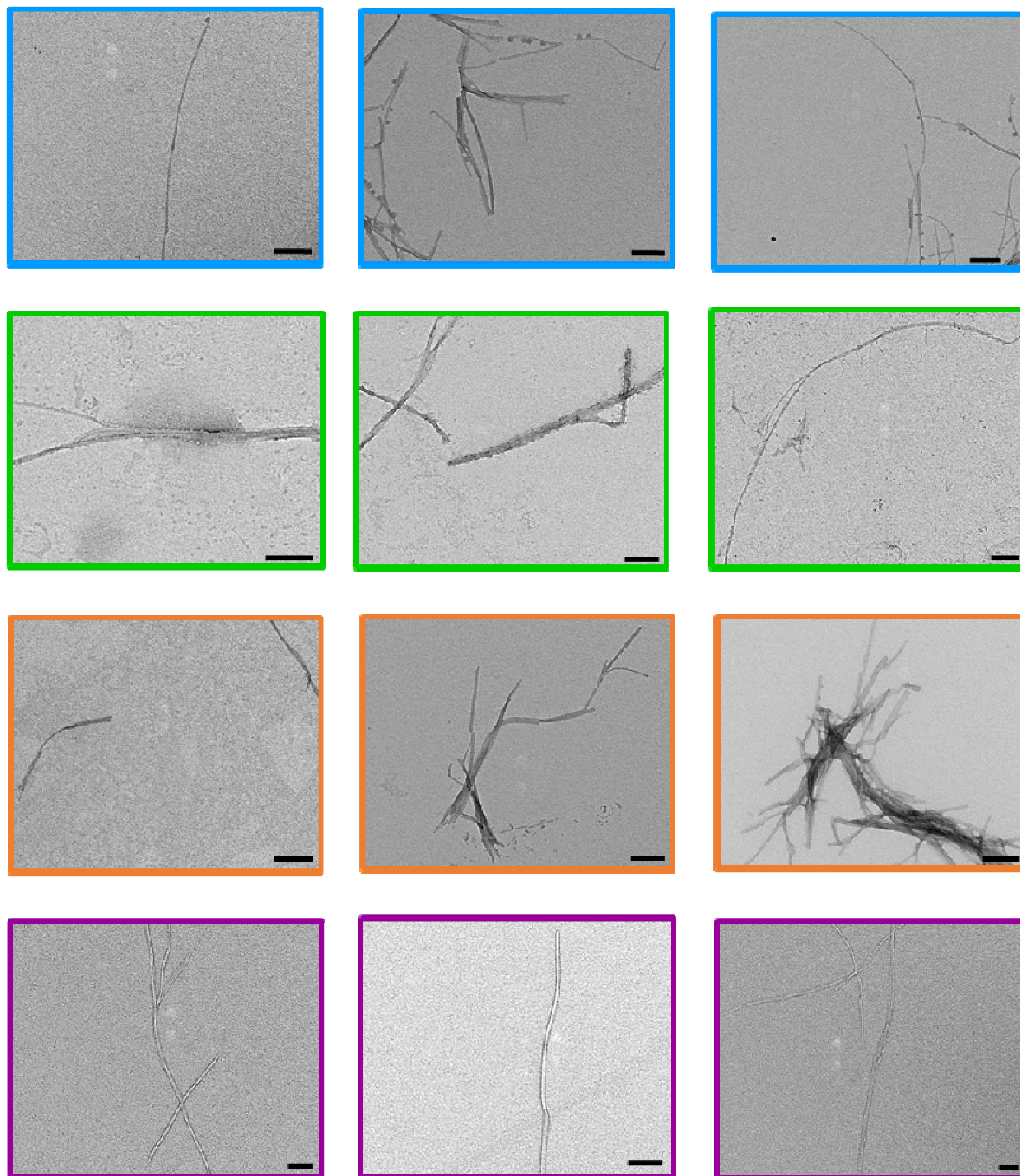

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637 **Supplementary Figure 4. Straight aSyn fibrils are formed during ThT assays from all purification**
638 **methods used.** aSyn samples from the ThT assays were imaged by TEM and show a straight fibril
639 morphology. The aSyn taken from wells of sample isolated by boiling (blue), (NH4)2SO4 precipitation
640 (orange) and periplasmic lysis (purple) were diluted to 5 μM before imaging. The aSyn isolated by
641 acid precipitation (orange) was incubated on the grid directly from the well at 20 μM.
642 Representative images are shown. Scale bar = 200 nm.

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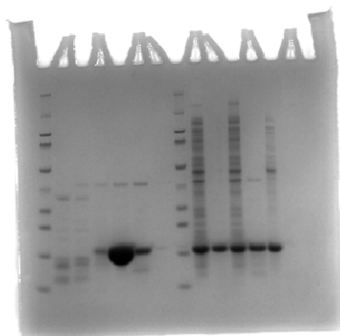
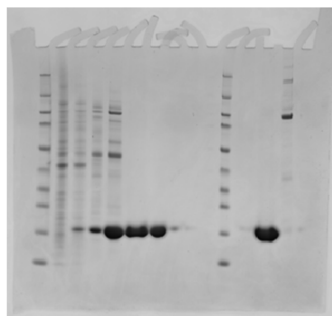


Figure 1a.II. and Supplementary Figure 1.



Supplementary Figure 1. and Figure 1d. vi.

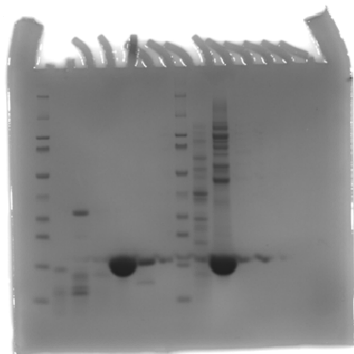
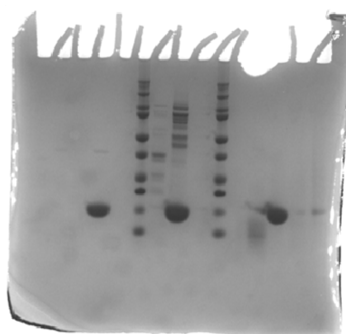


Figure 1b.II. and 1c.II.



Supplementary Figure 2j.

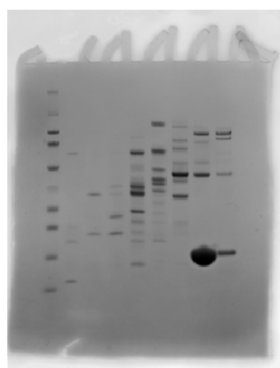
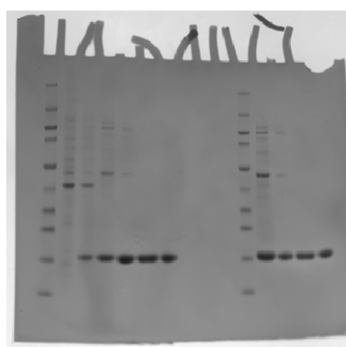


Figure 1d.II.



Supplementary Figure 2k.

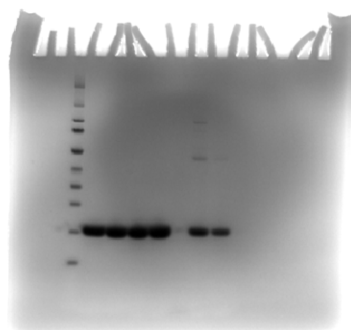


Figure 2a and Figure 1d.Iv.

646

647 **Supplementary Figure 5. All raw images of Coomassie blue stained SDS-PAGE gels used in**
648 **this publication.**