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Protein Precipitation Using Ammonium Sulfate

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Abstract

The basic theory of protein precipitation by addition of ammonium sulfate is presented and the most common applications are listed, Tables are provided for calculating the appropriate amount of ammonium sulfate to add to a particular protein solution.

Key terms for indexing

Ammonium sulfate; ammonium sulfate tables; protein concentration; protein purification

BASIC THEORY

The solubility of globular proteins increases upon the addition of salt (<0.15 M), an effect termed salting-in. At higher salt concentrations, protein solubility usually decreases, leading to precipitation; this effect is termed salting-out ((Green and Hughes, 1955). Salts that reduce the solubility of proteins also tend to enhance the stability of the native conformation. In contrast, salting-in ions are usually denaturants.

The mechanism of salting-out is based on preferential solvation due to exclusion of the cosolvent (salt) from the layer of water closely associated with the surface of the protein (hydration layer). The hydration layer, typically 0.3 to 0.4 g water per gram protein (Rupley et al., 1983), plays a critical role in maintaining solubility and the correctly folded native conformation. There are three main protein-water interactions: ion hydration between charged side chains (e.g., Asp, Glu, Lys), hydrogen bonding between polar groups and water (e.g., Ser, Thr, Tyr, and the main chain of all residues), and hydrophobic hydration (Val, Ile, Leu, Phe). In hydrophobic hydration, the configurational freedom of water molecules is reduced in the proximity of apolar residues. This ordering of water molecules results in a loss of entropy and is thus energetically unfavorable. When salt is added to the solution, the surface tension of the water increases, resulting in increased hydrophobic interaction between protein and water. The protein responds to this situation by decreasing its surface area in an attempt to minimize contact with the solvent—as manifested by folding (the folded conformation is more compact than the unfolded one) and then self-association leading to precipitation. Both folding and precipitation free up bound water, increasing the entropy of the system and making these processes energetically favorable. Timasheff and his colleagues provide a detailed discussion of these complex effects (e.g., Kita et al., 1994; Timasheff and Arakawa, 1997).

It should be mentioned that the increase in surface tension of water by salt follows the well-known Hofmeister series, shown below (see Parsegian, 1995, and references therein). Hence, as an approximation, those salts that favor salting-out raise the surface tension of water the highest. As $(\text{NH}_4)_2\text{SO}_4$ has much a higher solubility than any of the phosphate salts, it is the reagent of choice for salting-out.

TIPS AND GUIDELINES

- With solid ammonium sulfate, a mortar and pestle can be used to break up any lumps.
- Use analytical grade as lower grade material is often contaminated with heavy metals.
- Addition of ammonium sulfate acidifies the solution so use at least a 50 mM HEPES or Tris buffer etc., 5mM EDTA can also be included.
- Add solid ammonium sulfate slowly with gentle stirring; allow to dissolve before adding more solid, try to prevent foaming.
- On-line calculators can be accessed to conveniently determine the amounts of solid ammonium sulfate required to reach a given saturation. For example, EnCor Biotechnology Inc., has an on-line calculator based on the equations given in this appendix: <http://www.encorbio.com/protocols/AM-SO4.htm>.
- Ammonium sulfate solution, 4.1M saturated at 25 °C can be purchased from Sigma-Aldrich and other suppliers.
- Note: In the literature sulfate is often referred to by UK spelling: sulphate.

COMMON APPLICATION

Concentration of Proteins

Because precipitation is due to reduced solubility and not denaturation, pelleted protein can be readily resolubilized using standard buffers. After concentration, the protein is well suited for gel filtration (*UNIT 8.3*) whereby the buffer can be exchanged and the remaining ammonium sulfate removed. Alternately, the protein can be dissolved in a nonprecipitating concentration of $(\text{NH}_4)_2\text{SO}_4$ (e.g., 1 M) and then applied to a hydrophobic interaction matrix (*UNIT 8.4*).

Protein Purification

Practical details of selective precipitation are presented in *UNIT 4.5*, and an example in the purification of interleukin 1 β is given in *UNIT 6.2* where the protein is fractionated between ~50 – 77% saturation. Low molecular weight proteins, like interleukin-1 β , as a rule require higher salt concentration for precipitation than larger molecular proteins, for example, large

multi-protein complexes can often be salted out with < 20% saturation. Another example (classic) is the precipitation of IgG from blood sera. The addition of 40 – 45% ammonium sulfate precipitates IgG which can be further purified by anion exchange chromatography. Salt precipitation has been widely used to fractionate membrane proteins (Schagger, 1994). Due to bound lipid and/or detergents, ammonium sulfate precipitates have lower density than protein-only precipitates. During centrifugation, these precipitates will often float to the top of tube rather than pelleting; the use of swing-out rotors is recommended. Crystallization is a traditional method of protein purification. Jakoby (1971) describes a general method that involves extracting $(\text{NH}_4)_2\text{SO}_4$ -precipitated protein with successively dilute $(\text{NH}_4)_2\text{SO}_4$ solutions at low temperature. Although there are several methods for removing contaminating nucleic acids from protein solutions including, for example, addition of 0.1% (w.v) polyethyleneimine, a simple and effective approach is to apply the protein to a small anion exchange column equilibrated with 0.4M ammonium sulfate, where the nucleic acids binds to the column and the protein is collected in the flow-through.

Folding and Stabilization of Protein Structure

As mentioned above, $(\text{NH}_4)_2\text{SO}_4$ and other neutral salts stabilize proteins by preferential solvation (Timasheff and Arakawa, 1997). Proteins are often stored in $(\text{NH}_4)_2\text{SO}_4$, which inhibits bacterial growth and contaminating protease activities. Protein unfolded by denaturants such as urea can be pushed into native conformations by the addition of $(\text{NH}_4)_2\text{SO}_4$ (Mitchinson and Pain, 1986). A practical application is the folding of recombinant proteins. For example, HIV-1 Rev expressed in *E. coli* was solubilized using urea, purified by ion-exchange chromatography in the presence of urea, then folded by the addition of 0.5 to 1.0 M $(\text{NH}_4)_2\text{SO}_4$ (Wingfield et al., 1991).

Basic Calculations

Basic definitions—Percentage (%) saturation concentration of $(\text{NH}_4)_2\text{SO}_4$ in solution as % of maximum solubility at the given temperature. For example, at 0°C, a 100% saturated solution is 3.9 M.

Specific volume (sp. vol.) volume occupied by 1 g of $(\text{NH}_4)_2\text{SO}_4$ (ml/g) = inverse of density. At 0°C, if 706.8 g of $(\text{NH}_4)_2\text{SO}_4$ is added to 1 L of water the volume = 1000 ml + volume occupied by the salt (706.8×0.5281 ml) = total volume of 1373.26 ml. The molarity = 3.9 M.

Calculating quantities of $(\text{NH}_4)_2\text{SO}_4$ to be added—By weight. The following equation is used to calculate the weight of solid $(\text{NH}_4)_2\text{SO}_4$ to be added to 1 liter of solution of initial concentration S_1 to produce final saturation S_2 :

$$\text{weight(g)} = \frac{G_{\text{sat}}(S_2 - S_1)}{1 - (PS_2)}$$

where:

G_{sat} = grams of $(\text{NH}_4)_2\text{SO}_4$ contained in 1 liter of saturated solution. For example, at 0°C , $G_{\text{sat}} = 515.35$ (see Table A.3F.1).

S_1 and S_2 are fractions of complete saturation; for example, a 20% saturation is expressed as 0.2.

$$P = (\text{sp. vol.} \times G_{\text{sat}}) / 1000$$

For example, $P = 0.2722$ and 0.2945 at 0°C and 25°C , respectively.

By volume. The following equation is used to calculate volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution to be added to 100 ml of solution to increase saturation from S_1 to S_2 :

$$\text{volume(ml)} = \frac{100(S_2 - S_1)}{1 - S_2}$$

For example, to raise 100 ml of 0.2 saturated solution to 0.70 saturation:

$$100\text{ml}(0.2) + x\text{ml}(1.0) = (100 + x\text{ml})0.70$$

$$20 + x = 70 + 0.7x$$

$$x = 166.66\text{ml}$$

Hence, 166.66 ml of saturated solution is added to 100 ml of 20% saturated solution to give 266.66 ml of 70% saturated solution.

AMMONIUM SULFATE TABLES

The tables shown are taken from Wood (1976). Table A.3F.2 gives the weight of $(\text{NH}_4)_2\text{SO}_4$ to be added to a solution to obtain the desired concentration. Table A.3F.3 gives the volume of a 3.8 M solution to add to obtain a desired concentration. Tables A.3F.4 and A.3F.5 give the final volumes after the addition of the solid salt or a 3.8 M solution, respectively. The concentration of $(\text{NH}_4)_2\text{SO}_4$ is expressed in molarity (corresponding % saturation is indicated in Table A.3F.2). The data is valid for solutions at 0°C , and the variation of specific volume with concentration is taken into account. For a table referring to solutions at 25°C , see Green and Hughes (1955).

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KEY REFERENCE

- Wood. 1976. See above.

Table A.3F.1Density and Molarity of Ammonium Sulfate Solutions^{a,b}

Temperature (°C)	0	10	20	25
(NH ₄) ₂ SO ₄ (g) added to 1 liter of water to give saturated solution	706.8	730.5	755.8	766.8
(NH ₄) ₂ SO ₄ (g) per liter saturated solution	515.35	524.60	536.49	541.80
Molarity of saturated solution	3.90	3.97	4.06	4.10
Density (g/ml)	1.2428	1.2436	1.2447	1.2450
Specific volume in saturated solution (ml/g)	0.5281	0.5357	0.5414	0.5435

^aMolecular weight of (NH₄)₂SO₄ = 132.14.^bAdapted from Dawson et al. (1986).

Table A.3F.2

Grams of Ammonium Sulfate to Add to 1 Liter of Solution at 0°C

Percent saturation	Initial molarity	Final molarity															
		0.00	0.20	0.40	0.60	0.80	1.00	1.20	1.40	1.60	1.80	2.00	2.20	2.40	2.60	2.80	3.00
0.0	0.00	0.00	26.7	54.0	81.9	111	140	170	202	234	267	302	338	375	413	453	495
5.1	0.20	0.00	0.00	27.0	54.7	83.0	112	142	173	205	238	272	308	344	383	422	464
10.3	0.40		0.00	0.00	27.4	55.4	84.2	114	144	176	209	243	278	314	352	391	432
15.4	0.60				0.00	27.7	56.2	85.5	116	147	179	213	247	283	321	359	400
20.5	0.80					0.00	28.1	57.1	87.0	118	150	183	217	252	289	328	368
25.7	1.00						0.00	28.6	58.1	88.5	120	153	186	221	258	296	335
30.8	1.20							0.00	29.1	59.1	90.2	122	156	190	226	264	303
35.9	1.40								0.00	29.6	60.2	91.9	125	159	194	231	270
41.1	1.60									0.00	30.2	61.4	93.7	127	162	199	236
46.2	1.80										0.00	30.7	62.6	95.7	130	166	203
51.3	2.00											0.00	31.3	63.9	97.7	133	170
56.5	2.20												0.00	32.0	65.2	99.8	136
61.6	2.40													0.00	32.7	66.7	102
66.8	2.60														0.00	33.4	68.2
71.9	2.80															0.00	34.2
77.0	3.00																0.00
82.2	3.20																0.00
87.3	3.40																0.00
92.4	3.60																0.00
97.6	3.80																0.00
100.0	3.90																0.00

Table A.3.F.3
 Milliliters of a 3.8 M Ammonium Sulfate Solution to Add to 1 Liter of Solution at 0°C

Initial molarity	Final molarity																	
	0.00	0.20	0.40	0.60	0.80	1.00	1.20	1.40	1.60	1.80	2.00	2.20	2.40	2.60	2.80	3.00	3.20	3.40
0.00	0.00	55.3	117	185	263	351	452	570	709	875	1077	1330	1655	2088	2693	3600	5111	8134
0.20		0.00	58.4	124	197	281	377	489	621	779	972	1213	1522	1933	2508	3371	4809	7684
0.40			0.00	61.9	132	211	302	408	534	683	866	1094	1387	1777	2322	3140	4503	7228
0.60				0.00	65.9	141	227	327	446	587	760	975	1252	1620	2135	2907	4194	6768
0.80					0.00	70.5	152	246	357	490	652	855	1115	1462	1946	2673	3884	6305
1.00						0.00	75.9	164	268	393	545	735	978	1303	1756	2437	3570	5837
1.20							0.00	82.3	179	295	437	613	840	1143	1565	2199	3255	5366
1.40								0.00	89.7	197	328	492	702	981	1372	1959	2936	4891
1.60									0.00	98.7	219	369	562	819	1179	1718	2616	4412
1.80										0.00	110	247	423	657	984	1475	2294	3931
2.00											0.00	124	282	494	789	1232	1971	3447
2.20												0.00	141	330	593	988	1645	2961
2.40													0.00	165	396	742	1319	2472
2.60														0.00	198	496	991	1981
2.80															0.00	248	662	1489
3.00																0.00	332	994
3.20																	0.00	498
3.40																		0.00

Table A.3.F.4
Final Volume in Millimeters After Addition of Solid Ammonium Sulfate to 1 Liter of Solution at 0°C

Initial molarity	Final molarity																				
	0.00	0.20	0.40	0.60	0.80	1.00	1.20	1.40	1.60	1.80	2.00	2.20	2.40	2.60	2.80	3.00	3.20	3.40	3.60	3.80	3.90
0.00	1000	1010	1023	1033	1046	1060	1074	1090	1106	1123	1142	1161	1181	1203	1225	1249	1275	1301	1329	1359	1373
0.20		1000	1011	1023	1035	1049	1063	1079	1095	1112	1130	1149	1169	1191	1213	1237	1262	1288	1316	1345	1359
0.40			1000	1012	1024	1037	1052	1067	1083	1100	1118	1137	1157	1178	1200	1223	1248	1274	1301	1330	1344
0.60				1000	1012	1025	1039	1054	1070	1087	1105	1124	1143	1164	1186	1209	1233	1259	1286	1315	1329
0.80					1000	1013	1027	1042	1057	1074	1091	1110	1129	1150	1172	1194	1218	1244	1271	1299	1313
1.00						1000	1014	1028	1044	1060	1077	1096	1115	1135	1156	1179	1203	1228	1254	1282	1296
1.20							1000	1014	1030	1046	1063	1081	1100	1120	1141	1163	1187	1211	1237	1265	1278
1.40								1000	1015	1031	1048	1065	1084	1104	1125	1147	1170	1194	1220	1247	1260
1.60									1000	1016	1032	1050	1068	1088	1108	1130	1152	1176	1202	1228	1242
1.80										1000	1016	1033	1052	1071	1091	1112	1135	1158	1183	1209	1222
2.00											1000	1017	1035	1054	1073	1094	1116	1140	1164	1190	1203
2.20												1000	1018	1036	1056	1076	1098	1121	1145	1170	1183
2.40													1000	1018	1037	1058	1079	1101	1125	1150	1162
2.60														1000	1019	1039	1060	1082	1105	1129	1142
2.80															1000	1019	1040	1062	1085	1109	1121
3.00																1000	1020	1041	1064	1087	1099
3.20																	1000	1021	1043	1066	1077
3.40																		1000	1022	1044	1055
3.60																			1000	1022	1033
3.80																				1000	1011
3.90																					1000

Table A.3.F.5
Final Volume in Millimeters After Addition of 3.8 M Ammonium Sulfate Solution to 1 Liter of Solution at 0°C

Initial Molarity	Final molarity																	
	0.00	0.20	0.40	0.60	0.80	1.00	1.20	1.40	1.60	1.80	2.00	2.20	2.40	2.60	2.80	3.00	3.20	3.40
0.00	1000	1051	1109	1174	1248	1333	1432	1547	1683	1847	2047	2298	2621	3051	3654	4560	6070	9091
0.20		1000	1055	1117	1187	1268	1362	1471	1601	1757	1947	2186	2493	2902	3476	4337	5773	8646
0.40			1000	1059	1126	1202	1291	1394	1517	1665	1846	2072	2363	2751	3294	4111	5472	8196
0.60				1000	1063	1135	1219	1317	1433	1573	1743	1957	2232	2598	3112	3883	5168	7741
0.80					1000	1068	1147	1239	1348	1479	1640	1841	2099	2444	2927	3652	4862	7282
1.00						1000	1074	1160	1262	1385	1535	1723	1966	2289	2741	3420	4552	6818
1.20							1000	1080	1176	1290	1430	1605	1831	2131	2553	3185	4240	6350
1.40								1000	1088	1194	1324	1486	1694	1973	2363	2948	3924	5878
1.60									1000	1097	1216	1365	1557	1813	2171	2709	3606	5402
1.80										1000	1108	1244	1419	1652	1979	2469	3287	4922
2.00											1000	1122	1280	1491	1785	2227	2965	4441
2.20												1000	1141	1328	1590	1984	2642	3956
2.40													1000	1164	1394	1740	2316	3469
2.60														1000	1198	1494	1989	2979
2.80															1000	1248	1661	2488
3.00																1000	1331	1994
3.20																	1000	1498
3.40																		1000