Partial purification of alkaline phosphate from potato and desalting of purified fraction.

Requirements

1. Potato tubers
2. Ammonium sulfate
3. Dialysis bag
4. Pipettes, Centrifuge tubes, Flasks etc

The salting-out technique of protein purification is mainly dependent on the hydrophobic character of the protein. The salt is dissolved into the solution containing the protein. Water will solvate the added salt ions, decreasing the solvation of the protein itself. This decrease in solvation exposes the hydrophobic
regions of the protein, which then interact with each other to form aggregates that will precipitate. For this laboratory experiment, we will use ammonium sulfate. The optimum concentration of ammonium sulfate required to precipitate the protein of interest is determined by adding increasing amounts of the ammonium sulfate and saving the precipitate for further analysis. A table is included in this handout for preparing protein solutions of different concentrations of ammonium sulfate. One disadvantage of this method is the high amount of salt that must be removed from the precipitate. To remove the salt from the protein sample, we will
use both dialysis.

Procedure:

Peeled and diced potato tubers (600 g) were homogenized in 300 ml of 50MmTris HCl pH 10.0.The homogenate was squeezed through six layers of cheesecloth and centrifuged at 14,000g for 20 min. Supernatant fractions were pooled and designated the crude extract. Store a small fraction of crude extract in -20°C.

Step 1: 0-30% ammonium sulfate cut

1. Pipet 100 mL of crude extract into a 250 mL flask labeled 0-30 (be sure to put your name on the flask as well).

2. Weigh out the required amount of ammonium sulfate for 30% saturation at 0 C (see table). The initial concentration of ammmonium sulfate is 0%.

3. Add ammonium sulfate slowly, swirl the flask and allow to dissolve. Repeat with the remaining portions of ammonium sulfate.

4. Place the centrifuge tube on ice for 15 minutes.

5. Centrifuge for 10 minutes at 10,000 x gravity. (Remember to balance the centrifuge tubes).

6. Label a flask “30-50”. Decant the supernatant from the previous step into the flask marked 30-50. Measure the volume and save for step 7. Dissolve the white precipitate (in centrifuge tube) in 1.0 mL of Tris HCl pH 8.0 and transfer this to a microcentrifuge tube marked “0-30% Cut”.

Step 2. 30-50% ammonium sulfate cut

7. Weigh out the required amount of ammonium sulfate for 50% saturation at 0 C (see table). Remember the initial concentration of ammonium sulfate is 30%.

9. Add the ammonium sulfate slowly as earlier steps

10. Repeat steps 4 and 5.

11. Label a flask “50-80”. Decant the supernatant from the previous step into the flask marked 50-80. Measure the volume and save for step 12. Dissolve the white precipitate (in centrifuge tube) in 1.0 mL of Tris HCl pH 8.0 and transfer this to a microcentrifuge tube marked “30-50% Cut”.

Step 2. 50-80% ammonium sulfate cut

12. Weigh out the required amount of ammonium sulfate for 80% saturation at 0°C (see table). Remember the initial concentration of ammonium sulfate is 50%.

9. Add the ammonium sulfate slowly as earlier steps

10. Repeat steps 4 and 5.

11. Discard the supernatant. Dissolve the white precipitate (in centrifuge tube) in 1.0 mL of 50MmTris HCl pH 10.0 and transfer this to a microcentrifuge tube marked “50-80% Cut”.

Step 3. Dialyze the following four samples against 50MmTris HCl pH 10.0.

a) Crude extract
b) 0-30% Cut.
c) 30-50% Cut
d) 50-80% Cut.

Final Concentration of Ammonium Sulfate: Percentage Saturation at 0°

Initial concentration of ammonium sulfate	Percentage saturation at 0°																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

Adapted from “Data for Biochemical Research” (R.M.C. Dawson, D.C. Elliott, and K.M. Jones, eds.), 2nd Ed. Oxford Univ. Press, London, 1969.

Dialysis
Dialysis is a procedure to separate smaller molecules (e.g., salt) from larger molecules (e.g., protein) by using a semipermeable membrane that allows the passage of the smaller molecules but not the larger molecules. In this lab we will use dialysis tubing that does not allow the passage of molecules exceeding 8,000 to 10,000 dalton in molecular weight. After 4-6 hours of dialysis, equilibrium is achieved, at which point the concentration of the dialyzable material (salt) is the same on the inside and outside of the dialysis bag. If the volume outside the bag is much larger than the volume inside the bag, there will be a substantial decrease in the salt concentration within the dialysis bag. If the outside solution is changed several times during the dialysis (normally morning, noon and night), an even greater decrease in the salt concentration can be achieved.

Procedure: Dialysis of the 0-50 ammonium sulfate precipitate

1. Soak a 20 cm x 1 cm piece of hydrated dialysis tubing in distilled water for five minutes. Tie a single knot at one end. Transfer the protein solution from the microcentrifuge tube marked "Dialysis" into the dialysis tubing with a plastic transfer pipette. Be sure to keep the tubing with the knotted end down.

2. Remove air above the solution in the tubing by running the tubing between your thumb and index finger.

3. Tie a second knot in the dialysis tubing above the solution allowing some head space (but no air) for the influx of water into the tube during the dialysis. Place the tube in a 1 L beaker containing the appropriate buffer (in this case water) and a stirring bar. Place the beaker on a magnetic stirring plate. Stir the solution at a high enough speed to ensure proper circulation of the water. Dialyze at 5 C.

4. Change the dialysis fluid morning, noon and night until the dialysis fluid tests negative for sulfate with a drop of barium chloride. The barium cation reacts with the sulfate anion to form an insoluble white precipitate. If a precipitate is formed, the dialysis fluid needs to be changed. Use 1.0 mL of dialysis fluid and 1 drop of saturated barium chloride.

NOTE: ***The samples will be used for following two experiments in coming weeks.***

1. The dialyzed sample will be used for SDD-PAGE in the next lab period
2. Estimated the Alkaline Phosphate activities of all four dialyzed samples and calculate the fold purification.