Purification of Acid Phosphatase from Wheat Germ

There are three major steps involved in the purification of macromolecules. First, you must disrupt the cell you are using as a source. Second, you must selectively purify the macromolecule away from contaminating molecules. Third, you must both prevent degradation of the macromolecule during the procedure and you must preserve the native structure of the macromolecule to be purified. In the purification of proteins, you want to separate that protein from other “contaminating” proteins, and you want to purify it in its native conformation with retention of enzymatic activity.

In this experiment, you purify the enzyme Acid phosphatase from wheat germ. Acid phosphatase removes phosphate groups from a variety of molecules under slightly acidic conditions. Wheat germ has been found to be a particularly good source of the enzyme. You first disrupt the cells using hypotonic shock; the Acid phosphatase will leak out of the cells. You then selectively purify the enzyme using a variety of precipitation steps, including a heating step that denatures most protein while leaving Acid phosphatase intact. The Acid phosphatase at the final stage of purification in this experiment is not pure, but could be purified further by standard biochemical techniques, including ion exchange columns, gel filtration, centrifugation, and so forth. You will use a synthetic substrate PNPP (para-nitrophenylphosphate) to follow the extent of purification of Acid phosphatase, and will do some characterization of the enzyme.

A frequent source of errors in this experiment is to calculate incorrectly the volumes of ammonium sulfate and MnCl₂ that you must add. Another major error is to incorrectly prepare dilutions of your enzyme for assays. Please double check your calculations at each step in the procedure.

For the last part of your experiment, you will need to design your own experiment. More on that part of the experiment will be describe later.

Materials

Day 1:
- Raw Wheat Germ
- Cheesecloth
- 1 M MnCl₂
- Saturated (NH₄)₂SO₄ (pH 5.5)
- Cold deionized water

Day 2:
- Water baths at 70°C
- 0.20 M EDTA (pH 5.7)
- Methanol (cold)
- Saturated (NH₄)₂SO₄ (pH 5.5)
- Cold deionized water

Day 3:
- 0.2 M EDTA (pH 5.7)
- Dialysis Tubing

Days 4 and 5:
- 1 M Na acetate (pH 5.7)
- 0.1 M MgCl₂
- 0.05 M PNPP (MW = 263) [131.5 mg/10 ml]
- Water bath 30°C
- 0.5 M KOH
- 1.0 mM K₂HPO₄ (day 5, only)
Methods

Notes:
(1) *Always* keep everything on ice!
(2) Store 0.5 ml aliquots of the supernatants in microfuge tubes for later analysis.

**Day 1: Acid phosphatase purification, part 1**

1-1) Mix together 40 ml of cold water and 10 grams of raw wheat germ. Stir occasionally for 30 minutes on ice.

1-2) Filter the suspension into a centrifuge tube through two layers of cheesecloth placed in a funnel. Squeeze the cheesecloth to get most of the liquid out.

1-3) Centrifuge the filtrate for 10 minutes at 8,000 rpm and 4°C.

1-4) Decant the supernatant into a graduated cylinder. Note the volume.

\[
\text{Volume of Supernatant I} = \]

1-5) Put 0.5 ml sample of Sup. I in a microfuge tube and store it in a -20°C freezer. Later, we will use it for assays.

1-6) Put the remainder of Supernatant I in a beaker, and then place the beaker in an ice bath. Stir gently with a magnetic stir bar. Add 2.0 ml of 1M MnCl\(_2\) per 100 ml of Sup I. Stir for 5 to 10 minutes.

\[
\text{Volume of MnCl}_2 = \]

1-7) Centrifuge the mixture for 10 minutes at 8,000 rpm at 4°C, to remove precipitated material.

1-8) Decant the supernatant into a graduated cylinder. Note the volume.

\[
\text{Volume of Supernatant II} = \]

1-9) Save a 0.5 ml sample of Sup. II at store it in the freezer.

1-10) Put Sup. II into a beaker and place it in an ice bath. **Slowly** (dropwise) add 50 ml of cold saturated (NH\(_4\))\(_2\)SO\(_4\) per 100 ml of Sup. II. (Use a 10 ml pipette and add over a period of 5-10 minutes while stirring the solution gently.) The solution is 33% saturated.

*Note:* You add the salt slowly to prevent co-precipitation. Co-precipitation happens when one part of the solution has a higher concentration of salt than another part. The higher part will precipitate proteins. If those proteins are in the middle of an aggregate, they will not redissolve when the solution is thoroughly mixed. The result is that some proteins might precipitate at a lower average salt concentration than they would if you add the salt slowly, giving erroneous results.

\[
(\text{NH}_4)_2\text{SO}_4 \text{ added} = \]

1-11) Stir the solution for an additional 10-15 minutes.

1-12) Centrifuge for 10 minutes at 8,000 rpm at 4°C.

1-13) Decant the supernatant into a graduated cylinder. Note the volume and freeze until the next lab.

\[
\text{Volume of Supernatant} = \]
Day 2a: Acid phosphatase purification, part 2a

2-1) Put the sup back into a beaker. Add 62 ml \((\text{NH}_4\text{)}_2\text{SO}_4\) per 100 ml sup. Add *slowly* with gentle stirring. The solution is now 60% saturated.

\((\text{NH}_4\text{)}_2\text{SO}_4\) added =

2-2) Place the beaker in a 70°C water bath. Bring the solution to 60°C while stirring gently, and hold at that temperature for 2 minutes.

2-3) Plunge the beaker into an ice bath. Stir gently until the solution reaches 6°C to 8°C.

2-4) Centrifuge for 10 minutes at 8,000 rpm at 4°C.

2-5) Decant the supernatant into a graduated cylinder. Record the volume of the supernatant. (Where is the enzyme now?) SAVE THE PELLET!!

Volume of Supernatant III =

2-6) Save a 0.5 ml sample of Sup. III and store it in the freezer. Discard the rest of Sup. III.

2-7) Completely resuspend the pellet obtained from Step in cold water. Use 1/3 the volume of Sup III, but no more than 20 ml of water. (Why should the precipitated protein resuspend?)

Volume of water added =

2-8) When an even suspension is obtained, transfer to a 40 ml centrifuge tube, and centrifuge for 10 minutes at 8,000 rpm at 4°C.

2-9) Decant the supernatant into a graduated cylinder. Note the volume.

Volume of Supernatant IV =

2-10) Save a 0.5 ml sample of Sup. IV and store it in the freezer.

2-11) Store the tubes containing samples of Sups. I-IV in the freezer.

Day 2b: Determine protein concentrations using the Warburg-Christian method

2-12) Make a 1:25 dilution of Sup IV by adding 60 µl of Sup IV to 1.44 ml of water.

2-13) Read \(A_{260}\) and \(A_{280}\) on the UV spectrophotometer:

\[ A_{260} = \]

\[ A_{280} = \]

2-14) Calculate \(A_{280}/A_{260}\). Determine the correction factor from the table passed out in class.

\[ A_{280}/A_{260} = \]

Correction Factor =
2-15) The \((A_{280})\times\text{(Correction Factor)} = \text{mg/ml protein for a 1:25 dilution of Sup IV.\) Multiply this value by 25 to get the mg/ml of undiluted Sup IV.\)

\[
\text{mg/ml Sup IV} = 
\]

**Day 2c: Acid phosphatase purification, part 2c**

2-16) Adjust the protein concentration of the rest of Sup IV to 4-5 mg/ml by adding an appropriate volume of cold water. If the final volume is over 60 ml, please check your calculations. Put in a beaker on ice and stir gently.

\[
\text{Final mg/ml Sup IV} = 
\]

\[
\text{Final volume Sup IV} = 
\]

2-17) For each ml of diluted Sup IV, add 0.12 ml 0.20 M EDTA and 0.05 ml saturated \((\text{NH}_4)_2\text{SO}_4.\)

\[
\text{Volume of EDTA added} = 
\]

\[
\text{Volume of (NH}_4)_2\text{SO}_4 \text{ added} = 
\]

2-18) Slowly add 1.75 ml of cold methanol for each ml of solution obtained at step 2-16 (final volume of Sup IV). Keep the solution stirring and on ice. Place in the freezer until next time. Be sure to label everything with your lab group’s initials.

\[
\text{Volume of methanol added} = 
\]

**Day 3: Acid phosphatase purification, part 3**

3-1) Unfreeze the sample and centrifuge at 8,000 rpm for 10 minutes at 4°C.

3-2) Decant the supernatant and discard. **SAVE THE PELLET!**

3-3) Resuspend the pellets in 3 ml (total for all the pellets, not for each pellet) of cold water.

3-4) Centrifuge at 8,000 rpm for 10 min at 4°C.

3-5) While centrifuging, prepare the dialysis tubing by performing steps 3-10 a and b. Let the tubing sit in distilled water for 30 or more minutes. You need enough water to cover the tubing.

3-6) Save **both** the supernatant and the pellet.

3-7) Resuspend the pellet again in 3 ml cold water.

3-8) Centrifuge at 8,000 rpm for 10 min at 4°C.

3-9) Decant the supernatant. Combine with the supernatant from step 3-6. Record the total volume. Discard the pellet. **Save 1.0 ml** of Sup V for assays.

\[
\text{Volume of Supernatant V} = 
\]

3-10) Dialyze Supernatant V against 0.5 liter of 5 mM EDTA overnight:

a) Prepare a 0.5 L solution of 5 mM EDTA from the 0.2 M EDTA stock solution.

b) Wear gloves while handling dialysis tubing.
c) Wet a 12 cm piece of 23 mm dialysis tubing with distilled H₂O. It should hydrate for at least 30 minutes.
d) Use a dialysis bag clamp to close one end.
e) Transfer Supernatant V to the dialysis tubing.
f) Put on another clamp at the top of the dialysis tubing.
g) Place in a flask or beaker containing the EDTA. Cover the flask or beaker with plastic wrap.
h) Either use a magnetic stir bar to stir overnight at 4°C, or let it sit for a week or more without a stir bar at 4°C.

Before dialysis, Sup V still has some (NH₄)₂SO₄ in it. What about after dialysis? Acid phosphatase is inhibited by Hg²⁺, Pb²⁺, Fe²⁺, Ag²⁺, Zn²⁺, and Cu²⁺, but stimulated by Mg²⁺. Also, proteases are often stimulated by Mg²⁺ and other divalent cations. Why do you think EDTA is in the dialysis buffer?

Day 4: Characterization of Acid phosphatase

You will be characterizing the enzymatic activity of the Acid phosphatase you have partially purified.

Acid phosphatase is also called 2-phosphoglycerol phosphatase. The activity is rather nonspecific, so that we don’t have to use 2-phosphoglycerol as a substrate. The assay reaction uses a synthetic substrate: p-nitrophenylphosphate (PNPP). It is hydrolyzed by Acid phosphatase, yielding p-nitrophenol (PNP). PNP is colorless at pH 5.6, but at pH 7.6 it is yellow. The amount of PNP formed can then be easily measured using a spectrophotometer.

When one measures enzyme activity, one must be sure that there is a linear relationship between the measure of activity (in this case the intensity of the yellow due to PNP) and the actual amount of enzyme present. Most assays are linear for some range of protein and substrate concentrations and for some limited amount of reaction time.

You will characterize the phosphatase activity of Sup VI at various reaction times. You will also measure the protein concentration and phosphatase activity of all the samples you saved during the purification. And, finally, you will perform an experiment to determine the Michaelis-Menten constant (Kₘ) of Acid phosphatase.

There are a lot of assays to perform on Day 4. To be able to finish this experiment in a reasonable amount of time, you should spend time, before you come to lab, calculating how much of each sample you are going to add at each step. You should have figured out all your dilutions, i.e. how much sample and diluent you should add at each step.

4-1) Carefully remove the sample from dialysis bag by removing a clip at the end of the dialysis tubing. Do this over a funnel placed on top of a graduated cylinder, or over a beaker. Pour the sample into the cylinder and note the volume of the dialyzed sample.

Volume of Supernatant VI =

A. Time Curve

4-2) Prepare the assay mixture by putting the following reagents into a 13 X 100 mm test tube:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 M Na acetate pH 5.7</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>0.1 M MgCl₂</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>0.05 M PNPP</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>1.30 ml</td>
</tr>
</tbody>
</table>

4-3) Vortex the assay mixture and place it in a test tube rack in a 30°C water bath for 10 min to warm the mixture to the optimum temperature for Acid phosphatase.

4-4) Set up 8 test tubes, 13 X 100 mm, that each contains 1.25 ml 0.5 M KOH.
4-5) Dilute an aliquot of Sup VI to 1/20 its original concentration (0.05 ml Sup VI + 0.95 ml H₂O).

**Note:** You will be making a mix of the enzyme, substrate, and buffer. At various time points, you will remove some of the mix for spectrophotometric analysis. This method assures that each sample had the same mixture of chemicals.

4-6) Add 0.2 ml of the *diluted* Sup VI to the assay mixture from step 4-2 and vortex. Take a “0” time point by removing, as quickly as possible, 0.25 ml of the assay and pipette it into one of the tubes containing KOH. (The “0” time point is a control). Replace the assay mix in the 30°C bath.

Note that the final volume will be 3.0 ml after you add Sup VI. (What are the final concentrations of the reagents?)

4-7) At times of 5, 10, 15, 20, 25, 30, and 35 min., remove 0.25 ml of the mix and add it to one of the tubes containing KOH. (This raises the pH, not only turning the solution yellow, but also stopping the reaction. Why?)

4-8) Read the Absorbance at 405 nm. Use the zero time point as a blank.

4-9) Plot the A₄₀₅ readings vs. time. Where is the assay linear for time?

4-10) Save all of Supernatant VI for later use.

**B1. Preparation of a Purification Table: Protein concentration**

4-11) Use the Warburg-Christian method, which you used in a previous experiment, to measure the protein concentration in each of the fractions that you have saved. For the following dilutions, make 1000 µl:

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>Dilution</th>
<th>Volume</th>
<th>H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1/500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1/100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1/100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1/25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>1/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>1/5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If the absorbance is over 1.5, dilute the sample and read it again.
Day 5: Acid phosphatase kinetics

B2. Preparation of a Purification Table: Assays

5-1) For steps 5-1 through 5-5, you will use your Acid phosphatase assays to determine the amounts of the enzyme in your supernatants.

Prepare the following dilutions of each supernatant to determine the phosphatase activity (you need at least 0.3 ml final volume for each fraction):

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>Dilution</th>
<th>Volume</th>
<th>H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1/100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1/100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1/20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1/100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>1/50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>1/25</td>
<td></td>
<td></td>
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</tbody>
</table>

5-3) Prepare one assay reaction for each fraction, plus a control containing no protein. You can prepare a mix containing the chemicals below. Because of pipetting errors, prepare a mix for eight tubes (you use seven tubes). Aliquot 0.3 ml of the mix out to each of the seven tubes.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 M Sodium acetate</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>0.1 M MgCl₂</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>0.20 ml</td>
</tr>
</tbody>
</table>

5-3) To each of the assay reactions, add 0.15 ml of either the appropriate diluted fraction (from step 5-1, one per tube) or H₂O (as the control). Therefore, six of the tubes will each contain one of the supernatants, the seventh tube contains no enzyme (water).

5-4) You will now perform the assays. In order to ensure that each sample incubates for the same amount of time, samples will be started and stopped in intervals. For example, sample 1 will start at time 0 and end at time 15, sample 2 will start at time 1 and end at time 16, etc.

Therefore, at one minute intervals, to each tube successively add 0.05 ml PNPP, vortex, and immediately place in a 30°C water bath.

5-5) Starting at 15 minutes, remove one sample per minute (i.e., exactly 15 minutes for each sample), add 2.0 ml 0.5 M KOH.

5-6) Read the A₄₀₅. Use the no protein control as the blank.

5-7) For the calculations, assume a value of 18.8 X 10⁻³ μM⁻¹ cm⁻¹ (18.8 X 10⁻³ M⁻¹ cm⁻¹) as the molar extinction coefficient of PNP. Use this to calculate the amount of PNP, in micromoles, in each tube. Use Beer’s Law: C = A / εb, where C is concentration of PNP, b is the path length of light (i.e., the width of the tube: assume it is 1 cm), and ε is the molar extinction coefficient, and A is the absorbance (A₄₀₅) of the sample.

Be sure to calculate the total amount of PNP in the tube, not just the concentration.

5-8) Construct a purification table for Acid phosphatase. For each purification step, the table should include the total protein, total enzymatic activity, specific activity, percent recovery, and fold purification. The total activity is the
units of activity that you would see if you assayed all of your protein. The specific activity of each fraction is the total activity/total protein and is a measure of how pure the enzyme is. If X is the purification step, the percent recovery is \(100 \times \frac{\text{total activity in } X}{\text{total activity of Sup I}}\) and tells you how much of the enzyme that you started with remains in your purification. The Fold purification is \(\frac{\text{specific activity of } X}{\text{Sp. Act. of Sup I}}\), giving you a measure of how much you have purified the protein.

C. Determination of the Michaelis-Menten constant

5-9) Using a stock of 50mM PNPP, prepare at least 0.2 ml each for a set of dilutions of PNPP at the following concentrations:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25 mM</td>
</tr>
<tr>
<td>2</td>
<td>8.0 mM</td>
</tr>
<tr>
<td>3</td>
<td>4.0 mM</td>
</tr>
<tr>
<td>4</td>
<td>2.0 mM</td>
</tr>
<tr>
<td>5</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>6</td>
<td>0.50 mM</td>
</tr>
<tr>
<td>7</td>
<td>0.25 mM</td>
</tr>
<tr>
<td>8</td>
<td>0.0 mM</td>
</tr>
</tbody>
</table>

5-10) Mix 1 ml of 1.0 M Na acetate and 1 ml of 0.1 M MgCl\(_2\) (“Na+Mg”).

5-11) Make 1 ml of a dilution of an aliquot of Sup VI (to 1/10 its original concentration.). Dilute the stock K\(_2\)HPO\(_4\) solution to 1 mM.

5-12) Prepare two sets of 8 tubes each as follows:

- For each tube of Set A, mix 0.05 ml Na+Mg, 0.1 ml H\(_2\)O, and 0.05 ml of the appropriate PNPP dilution from step 5-9 (i.e., tube A-1 has dilution 1 from step 5-9, tube A-2 has dilution 2, etc.).
- For each tube of Set B, mix 0.05 ml Na+Mg, 0.1 ml 1 mM K\(_2\)HPO\(_4\), and 0.05 ml of the appropriate PNPP dilution from step 5-9 (i.e., tube A-1 has dilution 1 from step 5-9, tube A-2 has dilution 2, etc.).

**NOTE:** The difference between Sets A and B is the substitution of potassium phosphate for water. Each of the 16 tubes should contain 0.2 ml.

5-13) At one minute intervals, add 0.05 ml of the Sup VI dilution to each tube, vortex, and place in a 30°C bath. What is the final PNPP concentration in each reaction after the addition of Sup VI? (Final vol. = 0.25 ml)

5-14) After 15 minutes for each tube, add 1.25 ml 0.5 M KOH to each tube and vortex.

5-15) Read the \(A_{405}\) for each tube using the control as the blank.

5-16) Calculate the amount of PNP, in micromoles, produced for each sample.

5-17) Plot the inverse of the amount of PNP produced (1/\(v\)) vs. the inverse of the PNPP concentration (1/[\(S\)]) for each set of tubes. This is a Lineweaver-Burke plot. The x-intercept is \(-\frac{1}{K_m}\) where \(K_m\) is the Michaelis-Menten constant, the y-intercept is \(1/V_{max}\), and the slope of the line is \(K_m/V_{max}\).

5-18) Save all the supernatants for later analysis of their purity by gel electrophoresis.
Is the $K_m$ higher or lower when phosphate is present? Does this mean that phosphate enhances or inhibits phosphatase activity? What about the $V_{\text{max}}$? Is phosphate a competitive, noncompetitive, or uncompetitive inhibitor (enhancer)?

**Lab Questions**

1. During the purification of Acid phosphatase, you determined the protein concentration by the Warburg-Christian method. What were your $A_{260}$ and $A_{280}$ readings for Sup IV? What was the protein concentration? Why did you read the $A_{260}$?

2. Plot the $A_{405}$ vs. time for Sup VI on a sheet of graph paper. Why do you need to do a time course for the protein? Where is the plot linear? Where the curve is not linear, explain why this might have happened.

3. Why perform a time curve? What happens when you do a Lineweaver-Burke plot if you use conditions from the non-linear part of the curve?

4. Construct a purification table, include the step of purification, volumes, total protein, total activity, specific activity, fold-purification, and percent recovery for each fraction (see the chart on the next page). Show some sample calculations. Explain any anomalies in the table.

5. Why are specific activity and fold purification important in protein purification?

6. Plot $1/v$ vs. $1/[S]$ for your Sup VI in the presence and absence of phosphate. What are the $K_m$ and $V_{\text{max}}$ with and without phosphate? What type of inhibitor is phosphate? Explain. Be sure to plot +/- inhibitor on one graph.

7. Report all the details of your own experiment. What were the hypotheses? What were the results? What were the conclusions?

**References**


# Purification Table

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total volume (ml)</th>
<th>Protein conc. (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (Units)</th>
<th>Specific activity (U/mg)</th>
<th>Fold purification</th>
<th>Percent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract (Supernatant I)</td>
<td></td>
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<td>MnCl₂ precipitation (Supernatant II)</td>
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<tr>
<td>(NH₄)₂SO₄ precipitation (Supernatant III)</td>
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<tr>
<td>Resuspension and wash (Supernatant IV)</td>
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<td>Methanol precipitation (Supernatant V)</td>
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<tr>
<td>Dialysis (Supernatant VI)</td>
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</table>

**Definitions**

- Units = μmoles of PNP produced in 15 minutes at 30°C
- Specific activity = Total activity / Total protein
- Fold purification = Specific activity of the step / Specific activity of Supernatant I
- Percent recovery = 100 x Total activity of the step / Total activity of Supernatant I