EXPERIMENT 6: Sequence Determination of a Dipeptide

Day 1: Acid Hydrolysis of the Peptide and Preparation of the DNP-Derivatized Dipeptide
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1. Obtain duplicate samples of the unknown dipeptide, each containing about 2 mg. One sample will be in a microfuge tube and the other will be in a 13 x 100 mm glass test tube. Record the number of your unknown sample in your notebook.

2. To the sample in the glass test tube, add the following:

   200 µl distilled water
   100 µl of 4.2% NaHCO₃
   400 µl of FDNB reagent

3. Allow the sample to incubate for 1 hr at room temperature, mixing gently every 3-4 min. During this hour, check the pH every 10 min by dropping a spot of the reaction solution on pH paper. Maintain the pH at ~9.0 by adding drops of 4.2% NaHCO₃ as needed. Proceed with steps 4 and 5 during this 1-hr incubation.

4. To the sample in the microfuge tube, add 200 µl of 6N HCl. After mixing gently by pipetting up and down, use the same P-200 tip to transfer the acid-dissolved dipeptide from the microfuge tube to a 4-ml glass vial and seal with a Teflon-lined screw cap.

5. Using a marker, label the glass vial on the side with your group initials and place it in a 110°C oven overnight. Sample vials will be placed in a tray of sand to enhance heat distribution. DO NOT PUT ADHESIVE TAPE ON THE VIALS AS IT WILL BURN OFF.

6. Following the 1-hr incubation, add 1 ml of distilled water and 0.5 ml of 4.2% NaHCO₃ to the sample in the glass test tube. Make sure that the pH of the solution is above 8.0 before continuing.

7. Add an equal volume (~ 2 ml) of ether with a transfer pipet and mix with the same pipet. Allow the aqueous and ether phases to separate, remove and discard the ether (upper) phase containing excess (unreacted) FDNB with a transfer pipet.

8. Repeat Step 7 two more times (3 times, total) or until the ether phase no longer shows any yellow color.

9. To the resulting aqueous phase (containing the DNP-dipeptide), add 100 µl of 6N HCl. Make sure that the pH is ~1.0 by putting a small drop of the reaction on pH paper after mixing. You may notice some bubbles forming in the solution as the H⁺ causes the formation of CO₂ gas and water from the formation of H₂CO₃.

10. Extract the acidified, aqueous solution with 2 ml of ether as in Step 7. After mixing, allow the phases to separate, remove the ether phase (containing the DNP-dipeptide) and transfer to a 13 x 100 mm glass test tube. DO NOT discard the ether phase and DO NOT transfer ANY aqueous phase with the ether layer. Water dries MUCH slower than ether.

11. Repeat Step 10 two more times (3 times, total). The ether phases from all three extractions can be saved in the same test tube. The aqueous phase can be discarded.
12. In the fume hood, evaporate the ether containing the DNP-dipeptide by placing the tube in a beaker of warm (not hot!) water and directing a gentle stream of N₂ over the solution (see Fig. 6-7). Continue with this procedure until a dry, yellow solid remains at the bottom of the tube.

13. Dissolve the dried DNP-dipeptide (yellow solid) in 0.5 ml of acetone and transfer the sample to a 4-ml glass vial.

14. Evaporate the acetone under N₂ in the fume hood and redissolve the dried sample in 0.5 ml of 6 N HCl.

15. Seal the vial with a Teflon-lined screw cap, label the side of the vial with your group initials, and place it in a 110°C oven overnight. Sample vials will be placed in a tray of sand to enhance heat distribution. **DO NOT PUT ADHESIVE TAPE ON THE VIALS AS IT WILL BURN OFF.**

**Day 2: Thin-Layer Chromatography of the DNP-Amino Acid and Paper Chromatography of the Acid-hydrolyzed Dipeptide**

**Thin-Layer Chromatography of the DNP-Amino Acid**

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1. Transfer contents of the 4-ml vial containing the acid-hydrolyzed DNP-labeled dipeptide (yellow sample) to a clean 13 x 100 mm glass tube.

2. Add 2 ml distilled water and 2 ml of ether to the glass test tube. Swirl for 10 sec, allow the phases to separate, and transfer the ether phase to a clean 13 x 100 mm glass test tube. Extract with 2 ml ether two more times (3 times, total) and combine the ether phases in the same glass test tube. This final ether extraction is done to separate the N-terminal DNP-amino acid from the underivatized amino acid. Remember that the N-terminal DNP-amino acid carries no charge at low pH (ether phase), while the C-terminal amino acid has a charged α-amino acid at low pH (aqueous phase). **DO NOT DISCARD THE AQUEOUS PHASE OF THE ETHER EXTRACTION.**

Transfer 0.2 ml of the aqueous phase (after extraction) to a 1.5-mL polypropylene microfuge tube. Place the open tube in the Speed-Vac evaporator in the back of the lab. The evaporator will be run until the samples are reduced to less than one-fourth the original volume (should be ~30 µL). If the samples dry completely, resuspend the dry residue in 20 µL of water.

3. Evaporate the ether extracts (containing your N-terminal DNP-amino acid) to dryness under N₂ as before.

4. "Activate" a silica-gel thin-layer chromatography (TLC) plate by heating for 5 min in a 100°C oven. This procedure drives off excess moisture from the plate so that the TLC separation is more reproducible. **GLOVES MUST BE WORN AT ALL TIMES WHEN HANDLING THESE PLATES.**

5. Use a pencil to softly draw a straight horizontal line across the plate, 2 cm from the bottom. **It is essential that you do NOT score through the silica gel to the plate underneath.** Beginning 1.5 cm from the left side of the plate, make short vertical marks 1 cm apart, which intersect the horizontal line that represents the origin. There should be 8 marks to indicate the origins where you will spot samples and standards. Be sure to softly write your group initials in the upper corner of the plate.
6. Carefully spot 5 µl of the DNP-amino acid standards (provided by the instructor) along the origin of the plate at the location of the vertical marks. To prevent diffusion of the sample be sure to spot 1 µl of the sample, allow it to dry, spot another 1-µl aliquot, allow it to dry, etc.

7. Dissolve the dried DNP-amino acid in 100 µl of acetone.

8. Using the same technique as described in Step 6, carefully spot a 5-µl aliquot of your sample along the same origin as the DNP-amino acid standards. In case you did not recover a sufficient amount of the sample in the last ether extraction, you should also spot a 10-µl sample next to this. **The sample spot should be yellow enough in color that you will be able to identify it once the plate is developed.**

9. When all of the spots have dried, place the TLC plate (sample side toward the bottom) in a TLC tank containing 1 cm of the mobile phase solvent [chloroform:t-amyl alcohol:glacial acetic acid (70:30:3)] and replace the tank cover (see Fig. 6-8). **Remember to make a map or drawing of the TLC plate in your notebook so that you will be able to identify which spot corresponds to which sample.**

10. Allow the mobile phase to migrate up the plate until the solvent front is about 1 cm from the top of the plate (~2 hr).

11. Remove the plate from the tank and immediately mark the position of the solvent front with a pencil. After the plate has dried, circle the position of all of the spots on the plate with a pencil. Make a photocopy of the plate for each partner using the lab copier.

12. For each spot on the plate, calculate a relative mobility value ($R_f$) thus:

$$R_f = \frac{\text{distance traveled by sample (cm)}}{\text{distance traveled by solvent front (cm)}}$$

If the sample spot showed some diffusion during development, use the center of the spot in determining the $R_f$ value. Also, if the solvent front migrated unevenly across the plate, measure the distance traveled by the sample (cm) relative to the distance traveled by the solvent front (cm) in each lane.

13. From the $R_f$ value of your unknown sample compared to the $R_f$ values of the DNP-amino acid standards, what is the identity of the N-terminal amino acid in your unknown dipeptide?

**Paper Chromatography of Acid-hydrolyzed Dipeptide**

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1. Recover the 4-ml vial containing your acid hydrolyzed dipeptide (colorless sample).

2. Obtain a 20 x 20 cm piece of 3 MM Whatman chromatography paper from the instructor and draw a straight horizontal line across the bottom using a soft lead pencil about 1 inch (2.5 cm) from the edge. This line will designate the origin where your samples will be spotted.

3. On both edges of the paper perpendicular to the origin, draw two straight vertical lines from top to bottom that intersect the origin 1 cm from either edge of the paper. Beginning 1.5 cm from the left edge, make short vertical marks 2 cm apart, which intersect the horizontal line that represents the origin. Be certain there are at least 9 spots. All of the samples will be spotted on the origin at one of the vertical marks between the two vertical lines.
4. Spot 3 µl of each of the amino acid standards along the origin. As with the TLC plate, spot each sample in 1-µl aliquots in order to keep the spot diameter small. Indicate the identity of each sample spot below the origin using a soft lead pencil.

5. Next to the standards, spot a 1-µl and a 3-µl sample of your unknown acid hydrolyzed dipeptide. Next to the samples of your unknown acid hydrolyzed dipeptide, spot a 2-µL and a 6-µL sample of your C-terminal amino acid (from the Speed-Vac). As before, spot 1 µL at a time to keep the spot concentrated at the origin. There is no need to save the remainder of the hydrolysate because we will not be performing the Edman degradation manually.

6. When all of the spots have dried, roll the paper into the form of a cylinder so that the spotted samples face INWARD toward the bottom of the paper (see Fig. 6-8). Staple the paper in three places (top, middle and bottom) avoiding any overlap of the paper on the edges.

7. Place the paper (origin side down) in a large jar containing ~ 1.5 cm of mobile-phase solvent [butanol:acetic acid:water (60:15:25)].

8. Cap the jar and allow the mobile phase to migrate up the paper until it reaches 1 in. from the top (~ 4-6 hr). The TA will remove the paper from the jar, mark the position of the solvent front with a pencil, and allow the paper to dry in a fume hood.

**Day 3: Analysis of the Paper Chromatogram**

**Analysis of Paper Chromatogram**

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1. Spray the paper chromatogram lightly with ninhydrin reagent and heat for 5 min in a 100°C oven. The amino acids will appear as blue or purple spots (except L-proline, which will appear yellow). Figure 6-9 shows the reaction that amino acids undergo with ninhydrin.

2. Circle the position of all spots with a pencil and note the color, which may give some clue as to the identity of the amino acid. Make a photocopy of the chromatogram for each partner using the lab copier.

3. Calculate the $R_f$ values of the amino acids in your unknown sample, as well as those of the amino acid standards, using the same procedure and equation as that used for the TLC plate.

4. Based on the $R_f$ values and colors of the spots present in your unknown sample compared to those produced by the amino acid standards, what two amino acids are present in your unknown dipeptide? You should be able to deduce the sequence of the dipeptide from the paper chromatography of your acid hydrolyzed dipeptide sample alone. The results of the chromatography of the aqueous sample from your ether extraction should confirm the identity of the C-terminal amino acid.

**Preparation of PTC-Amino Acids and HPLC Analysis**

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These procedures will not be performed.