

Lab Weeks 4 & 5 – Dipeptide Sequence Determination (revised 1/02; KRD)

Purpose: This lab exercise is intended to demonstrate the mechanism for identifying the amino acid constituents in an unknown dipeptide and determining their sequence (i.e., which amino acid is on the C-terminus vs. N-terminus of the dipeptide).

Part A: Tagging of the N-terminal amino acid in an unknown dipeptide

Part B: Identification of the two amino acids in an unknown dipeptide

Part C: Separation and identification of the hydrolyzed amino acids and N-terminal (i.e., DNP-labelled) amino acid

Key Concepts and Terms:

Laboratory Skills:

Background

The compound 1-fluoro-2,4-dinitrobenzene (FDNB) reacts with free amino, imidazole, and phenolic groups at neutral to alkaline pH's to yield the corresponding, colored dinitrophenyl (DNP) compounds. Thus, the N-amino group on all amino acids, the ϵ -amino group on lysine, the hydroxy group on tyrosine, and the imidazole group on histidine of all free amino acids, oligopeptides, and polypeptides react to form DNP derivatives. See p.119-122 in Biochemistry Second Edition by Voet and Voet.

The reaction of FDNB with a peptide proceeds by the attack of an uncharged α -amino group on the electron-deficient carbon atom adjacent to the fluorine atom. For this reason the reaction solution is buffered at about pH 9.0 with NaHCO_3 . The reaction proceeds with release of HF, so that additional NaHCO_3 may be required during the reaction. A strong base is not used because OH^- ions also react with FDNB to form phenol as a side product.

If the DNP derivative of a peptide is hydrolyzed in acid and, while acidified, extracted with ether, all nonpolar (uncharged) entities will be extracted into the ether phase while all polar components will remain in the aqueous phase. Thus, the nonpolar N-terminal DNP-amino acids and diDNP-amino acids (from N-terminal lysine, tyrosine, or histidine) will be removed into the ether phase because their carboxyl groups are protonated (i.e., uncharged). Exceptions to this are those amino acids that have polar side chain residues in acid such as DNP-arginine and DNP-cysteic acid. In contrast, any amino acids that are not the N-terminus in the DNP-peptide will have charged amino groups in acid solution after hydrolysis of the peptide and remain in the polar aqueous phase. This is also true for non-N-terminus amino acids that have side chains that react with FDNB. That is, ϵ -DNP-lysine, O-DNP-tyrosine and imidazole-DNP- histidine do not extract into ether from an acidic solution.

Once the N-terminal DNP-amino acid derivatives are separated from those amino acids or DNP-amino acid derivatives that are not the N-terminus amino acid, the DNP-derivatives and free amino acids may be identified by chromatographic techniques. These data will yield part of the sequence of the peptide under study.

In this 2-period experiment, an unknown dipeptide will be examined by the above procedure. In the first lab period, you will complete Parts A (tagging of the N-terminal amino

acid) and B (hydrolysis of the dipeptide). Begin part A first and then during the 1 hour incubation, you can complete part B. Part C will be completed during the second lab period.

Materials

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| Dipeptides | 6N HCl |
| DNP amino acid standards | FDNB solution |
| Amino acid standards | 4.2% NaHCO ₃ |
| Peroxide-free ether | Pasteur disposable pipettes |
| Bunsen burner | Ninhydrin spray reagent |
| butanol:acetic acid:water (4:1:1, v:v:v). | CHCl ₃ :t-Amyl alcohol:glacial HOAc (70:30:3) |
| TLC plates - cellulose and silica | Capillary tubes |
| hydrolysis vials | |

Week 1

Part A. *Preparation of DNP derivative of the N-terminus amino acid and hydrolysis of an unknown dipeptide.* Place 2 mg of the dipeptide in a 12-ml conical centrifuge tube and then add 0.2 ml of water, 0.05 ml of 4.2% NaHCO₃, and 0.4 ml of FDNB solution. Shake the resultant suspension frequently for one hour. (**WARNING: Do not pipette FDNB solution by mouth.**) Maintain the pH around 8-9 with additional 4.2% NaHCO₃. Use pH paper to monitor the pH of the reaction. If a large amount of precipitate develops, the pH is too low. The separation of a small amount of oil is not a cause for concern as long as you continue frequent shaking of the tube. After 1 hr, add 1 ml of water and 0.05 ml of NaHCO₃. Extract the suspension, vortexing 30 secs. to assure good mixing) with an equal volume of peroxide-free ether to remove the unreacted FDNB. (**WARNING: All extractions with ether should be done in the hood. Keep ether away from open flame!!!!**) Centrifuge (if necessary) to separate the layers, then remove the ether with a Pasteur disposable pipette fitted with a rubber bulb. Continue extractions until the ether layer is completely colorless. Then adjust to approximately pH 1.0 with 6 N HCl using pH paper (approximately 0.1 ml of acid). Extract the DNP-dipeptide with 2 ml of ether three times, pool the ether extracts in a test tube or hydrolysis vial and carefully evaporate the pooled ether extracts by placing the tube in a beaker of warm water and directing a stream of air over the surface. Ether must be evaporated in the hood!!! If ether does not extract the yellow material readily, check the pH again.

Add 0.2 ml of acetone to the dried DNP-dipeptide and transfer to a hydrolysis vial. Several transfers may be necessary. (As an alternative, the acidified ether extract may be dried directly in the hydrolysis vial. All ether must be removed before hydrolysis begins.) The dried DNP-dipeptide should be a yellow solid. Remove the acetone as described for the ether and add 0.5 ml of 6 N HCl. Label the vial with heat resistant tape and give it to the assistant. The labeled vial will be sealed by the assistant and placed in an oven overnight at 100 C. The hydrolysed derivatized dipeptide is **SAMPLE A** for the next lab.

You will also be given your dipeptide already hydrolysed as approximately 1 mg of dried amino acids in an Eppendorf tube. Please derivatize the amino acids with FDNB exactly as described above for the unhydrolysed dipeptide. The derivatized hydrolysed dipeptide is **SAMPLE B** for the next lab.

Part B. Hydrolysis of the Dipeptide . An Eppendorf tube with approximately 1 mg of the unknown dipeptide will be provided by the TA. Add 30 μ L of 6 N HCl to the dipeptide. Seal one end of a 100 μ L capillary tube by melting in a Bunsen burner flame. Mix and transfer the 30 μ L containing the dipeptide to the capillary tube. You can readily accomplish this by warming the sealed end of the capillary (melting point) tube over a low flame. Then invert the warmed tube over the HCl suspension of the dipeptide. As the air in the tube cools, the sample will be drawn up into the capillary. Tap the tube gently so that the column of liquid is well away from the open end. Seal the tube with the flame of a Bunsen burner. (It is important that the capillary be completely sealed. If adequate equipment is not available to do this, have a teaching assistant seal the capillaries. Prepare a duplicate sealed capillary if you have the slightest question about the quality of your capillary seal.) Label the capillary with heat-resistant tape. Place the sealed capillary in an oven at 100 C for 12 to 16 hrs. The hydrolysed underivatized dipeptide is **SAMPLE C** in the protocol for the next lab.

Week 2

Part C. Separation of hydrolyzed amino acids by TLC. You will prepare your samples for both cellulose and silica gel thin layer chromatography (TLC). The cellulose TLC may take longer than the silica gel TLC, so once your samples are prepared, please start the cellulose TLC first.

Sample A. After overnight heating, open the vial with **SAMPLE A**, the hydrolysed derivatized dipeptide, and transfer the contents to a test tube with a disposable pipette. Add 2 ml of water, and extract with 2 ml of ether three times. ALL ETHER EXTRACTIONS MUST BE DONE IN THE HOOD!!!! Do NOT discard the aqueous phase; it contains the underivatized C-terminal amino acid of your dipeptide. The aqueous phase is **SAMPLE A2**.

Concentrate the combined ether extracts in a test tube as before, by putting the test tube into warm water and directing a stream of air on it IN THE HOOD. After evaporating all ether, dissolve the DNP-amino acid in 0.5 ml of acetone. This is **SAMPLE A1** for silica gel chromatography.

Concentrate the aqueous phase, **SAMPLE A2**, by CAREFULLY evaporating the water by passing the test tube slowly through a Bunsen burner flame. Do not hold the tube in the flame. Do not point the tube at yourself or anyone else. Be careful not to char the sample when it goes to dryness. Add 50 μ L of water to the dried sample and resuspend by vortexing. This is **SAMPLE A2** for cellulose TLC.

Sample B. After overnight heating, open the vial with **SAMPLE B**, the derivatized hydrolysed dipeptide, and transfer the contents to a test tube with a disposable pipette. Add 2 ml of water, and extract with 2 ml of ether three times. ALL ETHER EXTRACTIONS MUST BE DONE IN THE HOOD!!!! You may discard the aqueous phase as both of the amino acids of your dipeptide should be derivatized.

Concentrate the combined ether extracts in a test tube as before, by putting the test tube into warm water and directing a stream of air on it IN THE HOOD. After evaporating all ether, dissolve the DNP-amino acids in 0.5 ml of acetone. This is **SAMPLE B** for silica gel chromatography.

Sample C. Scratch the capillary tube with a file at both ends and open the capillary at both ends by snapping off the closed ends. It is best to do this while holding the capillary in a paper towel. Force the contents into an Eppendorf tube. Rinse the capillary tube with water if necessary. This is **SAMPLE C** for cellulose TLC.

Cellulose TLC Chromatography. Take a cellulose thin layer plate which has been activated by baking at 100 C for at least 10 minutes and with a number 2 pencil, make a line about 2 cm from the bottom of the plate. Make a small vertical mark with the pencil every 1 cm along this line to make lanes for spotting your samples. Label the upper right hand corner of the plate with your group and section number. The amino acid standards should be available from the TA. The TA will draw on the board the order in which the amino acid standards and your samples should be spotted. In general, the amino acid standards will be spotted on either side of your samples A2 and C, which will be spotted in the middle lanes. The TA will demonstrate the correct method for spotting the chromatogram. Whenever you handle the chromatogram, keep it extremely clean. Wear disposable plastic gloves. Your fingerprints will show upon later staining of the chromatogram with ninhydrin. The spot should be kept very small (2-3 mm in diameter). The cellulose TLC will be developed in a tank with butanol:acetic acid: water (4:1:1). These solutions should have been placed in the tank and the tank closed for at least 30 min to allow saturation of the vapor in the tank with the solvent. To aid this saturation, a piece of Whatman paper has been soaked in the solvent and added to the tank. Each tank can hold two chromatograms, so Groups 1 and 2 will share one tank, Groups 3 and 4 the second, etc. Put your chromatogram into the tank so that the bottom rests next to the glass ridge in the center of the tank. Lean the top of the chromatogram against the wall of the tank. If your chromatogram is in the front, the bottom should be in front of the glass ridge and the top should lean against the front wall of the tank. Close the tank and allow the solvent front to rise to within a few centimeters from the top (about 1.5 hours). Then remove the chromatogram (plastic gloves), mark the solvent front with a soft pencil and allow the chromatogram to air-dry in the hood. When the chromatogram is completely dry, spray it lightly with the ninhydrin spray reagent. Locate the spots by heating the chromatogram at 100 C in an oven for 5 min. The amino acids will appear as blue or purple spots (except proline and hydroxy proline, which appear as yellow spots in this system). Circle all spots with a pencil and note the colors of the freshly developed spots, which sometimes provide a clue about the identity of a spot. Calculate the R_f values for your unknown spot or spots and standard amino acids. The R_f value is the ratio of the distance travelled by the center of the spot to the distance travelled by the solvent. All distances are measured from the line drawn on the bottom of the plate where the samples were spotted. Record the size of the spots, the distance travelled by the center of the spot, the R_f values and the colors of the spots for each of your samples in a table. Use this information to identify the amino acids in your dipeptide (**SAMPLE C**) and the amino acid at the C-terminus of your dipeptide (**SAMPLE A2**).

Silica Gel GEL TLC. On a silica gel thin-layer chromatographic sheet that has been "activated" by heating at 100 C in an oven for 10 min shortly before use, draw a light line with a number 2 pencil about 2 cm from the bottom. Don't gouge the silica gel or make a trough with the pencil. Make light marks every 1 cm along the line to divide it into lanes for spotting your samples. Label the upper right corner of the plate with your group and section number. The TA will give you the exact order in which the DNP-amino acid standards and your samples should be spotted on the chromatogram. Your samples (A1 and B) will be in the center lanes and

flanked on both sides by the DNP-amino acid standards. The samples should be applied in small spots along the line in the lane. Since the samples are in acetone, the spots can be kept small by blowing on the chromatogram to evaporate the acetone while spotting. The spots should be kept very small (1 mm in diameter). Spot the required amount of sample to give a clearly visible yellow spot by allowing each application to dry before applying another spot. Similarly, spot known DNP-amino acids at other positions on the plate.

The plate will be developed in a tank that has been equilibrated with chloroform: t-amyl alcohol: glacial acetic acid (70:30:3) as the solvent. Students will share tanks as described above. Allow the solvent front to rise to within a few centimeters of the top of the plate (about an hour), mark the solvent front with a number 2 pencil and air dry the plate in the hood. Record the size of the spots, the distance travelled by the center of the spot, and the R_f values of the yellow spots in a table. Use this information to determine the identity of the amino terminus of your dipeptide (**SAMPLE A1**) and the composition of your dipeptide (**SAMPLE B**).

Determine the amino acid residues present by comparing the R_f values observed with those of standard amino acids. In the same way ascertain what amino acid is N-terminal. For samples A1 and B, two yellow spots will usually be observed. One is free dinitrophenol; the other is the DNP amino acid. Dinitrophenol is colorless below pH 4.0(why?). Expose the chromatogram to HCl vapors from a container of 12 N HCl in the hood to determine which spot may be dinitrophenol. (If the DNP-amino acid spot is hard to find, examination under UV light may prove useful) From these data suggest a sequence for your dipeptide. Justify your conclusions by tabulating the R_f values for the constituent amino acids of your dipeptide, the C-terminal amino acid, and the DNP derivative of the N-terminal amino acid along with appropriate standard values obtained on the same chromatograms.

Consider the data from the cellulose TLC and the silica gel TLC and give the sequence of your dipeptide. Draw the structure of your dipeptide. Justify any inconsistencies in the cellulose and silica gel TLC data for your dipeptide.

Discussion. The compound FDNB has proven extremely effective in sequence determination on proteins and polypeptides. In this work, peptide pieces, originating from the intact protein as a result of acid or enzymatic hydrolysis, are first separated by electrophoresis or chromatography. Treatment of these peptides with FDNB, followed by hydrolysis and chromatography, yields the N-terminal amino acid in each peptide. These data in combination with knowledge of the total amino acid composition of each peptide; the specificities of any enzymes used in peptide formation; and the C-terminal amino acid (obtained, for example, by short-time carboxypeptidase treatment) of each peptide-often allow the determination of the original sequence of short peptides. Such an approach was first used by Frederick Sanger to elucidate the structure of the hormone insulin.

A valuable extension of the Sanger technique is the use of the reagent dimethylaminonaphthylsulfonyl chloride (dansyl chloride) to form the corresponding N-terminal dansyl-amino acid derivatives. These compounds are intensely fluorescent and hence can be detected on thin layer chromatograms in quantities 100 times smaller than can be detected of DNP amino acids.

The release of either DNP-amino acids or dansyl-amino acids from a peptide for chromatography requires acid hydrolysis, which destroys all of the remaining sequence of the peptide. It would be extremely valuable to have a means of cleaving a derivative of the N-terminal amino acid from the peptide without hydrolyzing other peptide bonds. With such a

technique one could determine the sequence of the peptide by merely repeating the derivatization and cleavage cycle over and over, removing one amino terminal residue at a time. Such a technique is provided by Edman's reagent, phenylisothiocyanate. This reagent reacts to form a phenylthiocarbamyl derivative of the free α -amino group, which cyclizes in anhydrous HF to cleave the amino terminal residue from the peptide without cleaving other peptide bonds. The derivative of the N-terminal amino acid can be converted to a stable phenylthiohydantoin (called a PITH-amino acid), which can be identified by chromatography. This technique has been automated for step-by-step sequence determinations.