December 2025

Protein Folding: In the Footsteps of Anfinsen

By Andrea van Bruggen-van der Lugt, ABE The Netherlands

ABE Master Teacher Fellowship Program

AMGEN® Biotech Experience

Scientific Discovery for the Classroom

AMGEN® Biotech Experience

Scientific Discovery for the Classroom

The projects designed by the 2023–24 ABE Master Teacher Fellows are a compilation of curricula and materials that are aligned with Amgen Biotech Experience (ABE) and prepare students further in their biotechnology education. These projects were created over the course of a 1-year Fellowship in an area of each Fellow's own interest. Each is unique and can be adapted to fit the needs of your individual classroom. Objectives and goals are provided, along with expected outcomes. Projects can be used in conjunction with your current ABE curriculum or as an extension.

As a condition of the Fellowship, these classroom resources may be downloaded and used by other teachers for free. The projects are not edited or revised by the ABE Program Office for content, clarity, or language except to ensure safety protocols have been clearly included where appropriate.

We are grateful to the ABE Master Teacher Fellows for sharing their work with the ABE community. If you have questions about any of the project components, please reach out to us at ABEInfo@edc.org, and we will be happy to connect you with the author and provide any assistance needed.

AMGEN Biotech Experience

Scientific Discovery for the Classroom

The Netherlands

Universiteit Utrecht

HOGESCHOOL UTRECHT

Protein Folding

In the Footsteps of Anfinsen

TIME FRAME: 10 hours

SUGGESTED AGE RANGE: 15–18, Upper Secondary

SUGGESTED COURSE OR CONTENT AREA:

Molecular modeling
Professional skills in STEM/Profiles in STEM

AUTHOR: Andrea van Bruggen-van der Lugt

PROGRAM SITE: ABE The Netherlands

SUMMARY OF PROJECT IDEA

The overarching theme of this project is **protein folding**. Depending on their prior knowledge, students begin with an introduction to protein chemistry, which the teacher can decide to include if necessary.

As part of a bioinformatics exercise, students explore the structure of the α-amylase protein using the 3D Viewer available in the RCSB Protein Data Bank. They are then introduced to Anfinsen's experiment, which demonstrated that a protein's amino acid sequence determines its three-dimensional structure.

Following in Anfinsen's footsteps, students conduct an experiment to investigate the unfolding and refolding of α-amylase, examining whether the enzyme can regain its unique native state through self-assembly.

Through a cooperative learning strategy, students gain a detailed understanding of the laboratory techniques used in the experiment, the α-amylase protein, and Anfinsen's findings.

Product Summary Cover Page	
My overall topic is… (1–3 sentences)	The overall topic of this ABE project is **protein folding**. Following in the footsteps of Anfinsen, students investigate in an *in silico* experiment the unfolding and refolding of a protein to study the self-assembly process, in which a protein sequence spontaneously forms a unique native state. In a bioinformatic exercise, students explore the beauty of proteins.
I want students/ participants to understand the self-assembly process of proteins.	Participants will have to understand (prior knowledge): ● The four levels of protein structure ● The basics of protein chemistry Participants will be able to: ● Work in the lab ● Cooperate
The reason why I wanted to pursue this is…	To teach students about the beauty of protein molecules and the fascinating process by which a protein finds its unique active state. In recent decades, significant progress has been made in predicting the 3D structure of proteins using AI models, culminating in a Nobel Prize in Chemistry in 2024. AI models open new possibilities for designing a diverse range of proteins, which can be applied in drug development, vaccines, and sensors.
Resources used or created	Depending on their prior knowledge, students begin with an introduction to protein chemistry, which the teacher can decide to include if necessary. If students require additional support, the teacher should prepare their own resources to provide direct instruction on protein chemistry and Anfinsen's experiment.
Skills or Standards addressed	● Collaboration: Working in groups, students will improve their communication and teamwork skills. ● Practical skills: Hands-on activities will help students develop practical skills in scientific experimentation and data collection. ● Computing skills: In bioinformatics exercises, students use the RSCB Protein Data Bank to explore the 3D structure of proteins
Assessments or Post-Surveys	● Poster presentation - detailed information about the used laboratory techniques, the protein α-amylase, and the Anfinsen experiment ● Writing a report on their experimental process and results

Activity 1: Introduction to Proteins

1. Overview

Depending on their prior knowledge, students begin with the introduction to protein chemistry, which the teacher can decide to include if necessary.

In this preparatory activity, students will learn about **amino acids**—the fundamental building blocks of proteins—and the three-dimensional structure of **proteins**.

2. Learning Goals

Students:

- Understand the role of **amino acids** as the building blocks of proteins.
- Explore the **3D structure** of proteins.

3. Assessed Outcome

Students will develop an understanding of **amino acids** and **proteins** and **protein folding** at the molecular level.

4. Key Vocabulary

Amino acids, side chains, proteins, 3D protein structure, primary, secondary, tertiary, quaternary, peptide bond, hydrogen bond, ionic bond, disulfide bridge, alpha-helix, beta-sheet

5. Materials & LabXchange Pathway(s)

Computers
Worksheet
Proteins - LabXchange
Protein Folding - LabXchange
Exploring the Hydrophobic Core - LabXchange
Protein Folding - LabXchange

6. Teacher Preparation

Share the document **"Introduction to Proteins"**.

If students need extra support, the teacher should prepare their own resources to teach Protein Chemistry

7. Lab Safety Considerations

Not applicable.

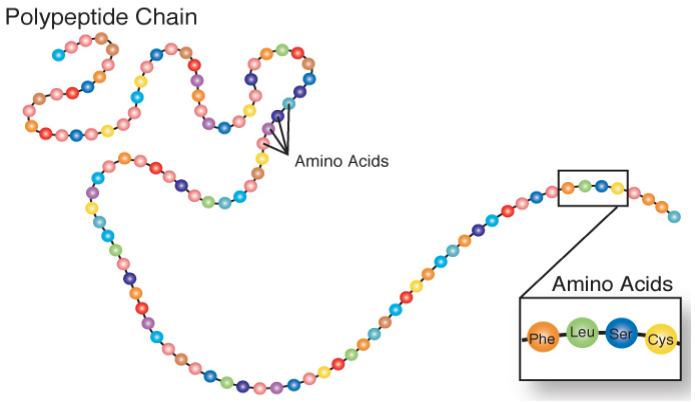
8. Sequence of Activities

Activity Description	*Time*	*Materials*
1. Preparatory task – Protein Chemistry	90 min	Document- Introduction to Proteins
2. Preparatory task – 3D-structure of Proteins	60 min	

Introduction to Proteins

Protein Chemistry

Proteins play essential roles in the body. They carry out most of the work within cells and are vital for the structure, function, and regulation of the body's tissues and organs. Proteins, also known as polypeptides, are large, complex molecules composed of hundreds or thousands of smaller units called amino acids, which are linked together in long chains.



Picture 1. A protein chain is like a chain of amino-acid beads (Protein Structure – Nutrition: Science and Everyday Application, v. 1.0)

A protein molecule is similar to a chain of beads, but in this case, the beads are tiny. First, we will examine the beads at a molecular level. Then, we will look at the chain formed when these beads are linked together.

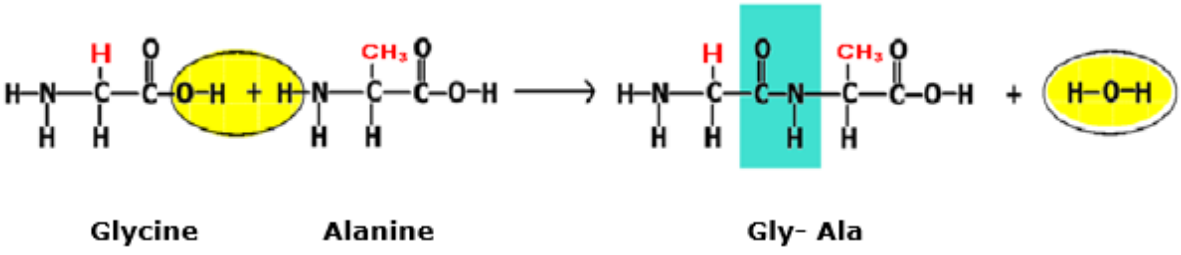
Let's take a closer look at the beads. The beads in the protein chain are amino acids.

Use *Proteins - LabXchange* – Figure 3.23 or Protein Folding - LabXchange to answer the following questions:

1. How many different amino acids are found in proteins?
2. What is the name of the largest amino acid? And what is the name of the smallest amino acid?
3. Examine the chemical structure of the amino acids that make up proteins. What do H, C, N, and O represent? And what do the lines between H, C, N, and O indicate?
4. What similarities and differences can be observed in their structures?

Amino acids share a common basic structure but differ in the composition of their variable side chain.

Through a chemical reaction, amino acids can bond to form a dipeptide, releasing water as a byproduct.



Picture 2. Formation of a dipeptide

In this example, glycine and alanine bond together to form a dipeptide

To represent the dipeptide formed by glycine and alanine, we use the three-letter code **Gly-Ala**.

1. Explain how **Gly-Ala** differs from **Ala-Gly**.
2. Design a small protein consisting of four amino acids. Start with four different amino acids by drawing the variable part (side chain). Include the name and three-letter code of each amino acid, beginning with **Asparagine**.

Picture 3: Four amino acids

3. How do you represent the peptide using the three-letter code when all four amino acids are linked together?
4. How many different sequences can be formed using these four amino acids? List all possible sequences using the three-letter code.

As more amino acids are added to the chain, it becomes a polypeptide.

There are over **100,000** different protein chains in the human body, each varying in length (number of amino acids) and amino acid sequence.

3D-Structure of Proteins

For proteins to function properly, they need to be correctly folded. The three-dimensional structure of each protein is based on the amino acid sequence of the protein.

Read *Proteins - LabXchange* – Figure 3.23 or Protein Folding - LabXchange and answer the following questions:

The chemical nature of the side chain determines the amino acid's nature (that is, whether it is acidic, basic, polar, or nonpolar).

1. *Describe the chemical character of the side chains of amino acids for each of the categories (acidic, basic, polar, nonpolar)*
2. *What type of binding do you expect between amino acids of each category (hydrogen bonds, van der Waals interactions, electrostatic interactions)*
3. *Which categories of amino acids would you expect to find on a soluble protein's surface and which would you expect to find in the interior?*
4. *What distribution of amino acids would you expect to find in a protein embedded in a lipid bilayer?*

Use the simulator Exploring the Hydrophobic Core - LabXchange to select different proteins and choose which parts of the protein molecule to highlight. Click and drag the molecule to rotate it. Observe where the polar and nonpolar parts of the protein end up and how they influence the overall structure of the larger protein molecule.

Use the simulator Protein Folding - LabXchange to explore the role of hydrophobic and hydrophilic interactions in protein folding. Generate a random peptide string and watch how it folds in an aqueous environment. Change the solvent to see what happens if the peptide is in an oily environment or a vacuum instead of an aqueous environment.

To understand how the protein gets its final shape or conformation, we need to understand the four levels of protein structure: primary, secondary, tertiary, and quaternary.

Watch the video What is a Protein? to get an overview of the structure of amino acids, the four different structural levels of protein, and examples of different types of protein in the body.

Read *Proteins - LabXchange* – Figure 3.25, 3.28, 3.29 and 3.30 or Protein Folding - LabXchange and answer the following questions

1. *What is the primary structure of a protein?*
2. *Why is the sequence of amino acids important for the function of a protein?*
3. *What are the two main types of secondary structures in proteins and how are they formed?*
4. *What does the tertiary structure of a protein involve?*
5. *What types of interactions can stabilize the tertiary structure of a protein?*
6. *Can you give examples of proteins with a quaternary structure?*

Activity 2: The Art of Protein Folding

1. Overview

In this bioinformatics activity, students will explore the **3D structure of proteins**, focusing on **α-amylase**.

2. Learning Goals

Students:

- Learn that information about protein structures is available in an online protein database (RSCB **PDB**).
- Use **PDB** to visualize proteins in different structural representations.
- Identify the **active center** of proteins at both the **micro** and **meso** levels.

3. Assessed Outcome

Students will use **PDB** to visualize **α-amylase** in different representations and investigate its structural characteristics.

4. Key Vocabulary

Ball-and-stick model, cartoon model, molecular surface model, space-filling model, C-terminus, N-terminus, active site, backbone, side chains, amino acids, protein database, 3D structure.

5. Materials & LabXchange Pathway(s)

Computers

6. Teacher Preparation

Share the document **"The Art of Protein Folding – 3D Structure"**.

7. Lab Safety Considerations

Not applicable.

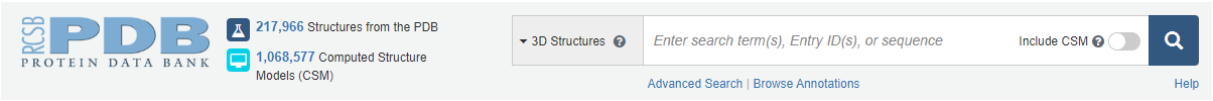
8. Sequence of Activities

Activity Description	*Time*	*Materials*
Bio-informatics activity	90 min	Document - the art of protein folding -3D structure

The Art of Protein Folding – 3D Structure

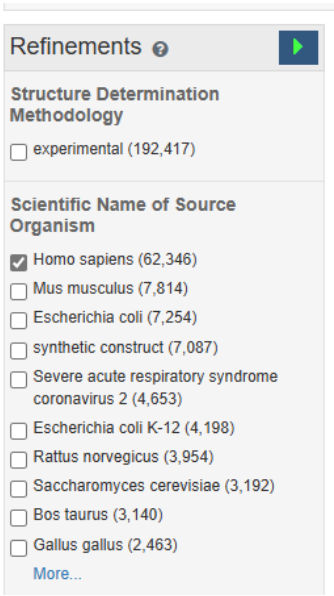
Parts of this exercise have been adapted and edited from the workshop '3D ontdekkingstocht in de wereld van eiwitten' by Dr. Andy Thunissen, Groningen, University, given at the NIBI conference 2017, 8106cc9f58fd01d3bed0f18813a5d97342b7ec2a.docx.

1. Open the Chrome browser and find the RCSB Protein Data Bank website. At the top, you will see the following information and a search tool:



   You can search using a description of the protein (in English) or a unique identifier called the PDB ID. If you find an interesting protein, note down the PDB ID to easily find it later.

Let's search for the α-amylase protein.



2. Type 'alpha-amylase' in the search bar and click 'Go.'

   How many protein structures do you find?

   On the left side of your screen, you can filter the results by the organism in which the proteins are found.

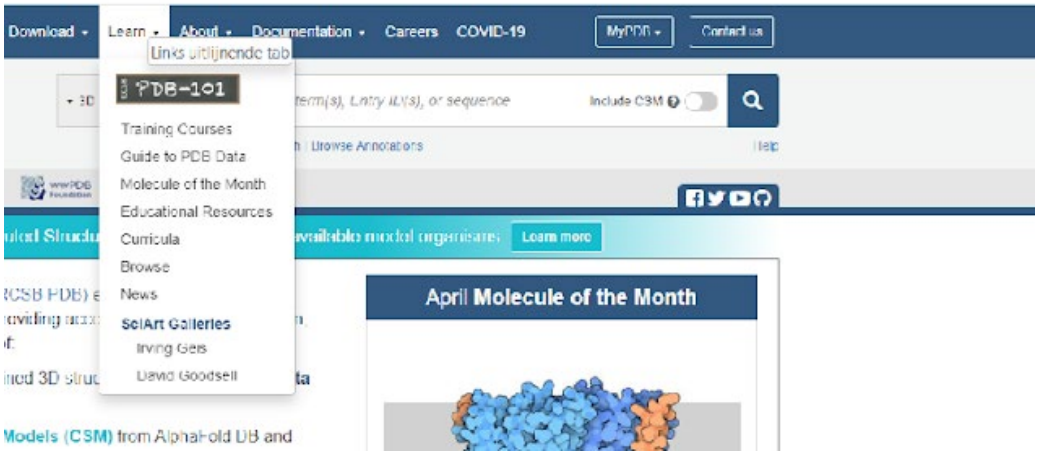
3. Select human α-amylase and click **Refinements**.

   Several structures of the α-amylase protein are displayed on the right side of the screen, each with a unique PDB ID. The names of the researchers who studied the 3D structure of the α-amylase protein are also mentioned.

4. Click on one of the PDB IDs to view the protein's 3D structure. Scroll to find information about the protein, its sequence, ligands, and more.

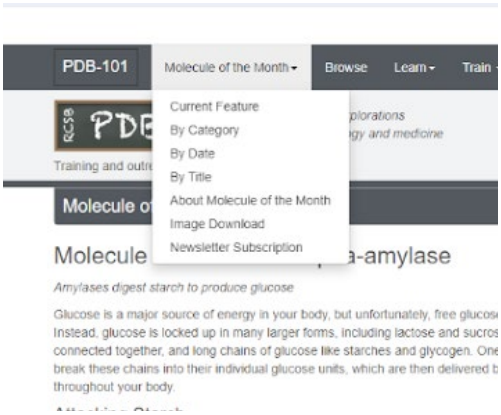
Continue exploring the α-amylase protein:

5. Click **LEARN – PDB 101** at the top of the webpage.



This is the educational section of the protein database. Use the various links to explore a wealth of interesting information.

One section is **'About Molecule of the Month.'**



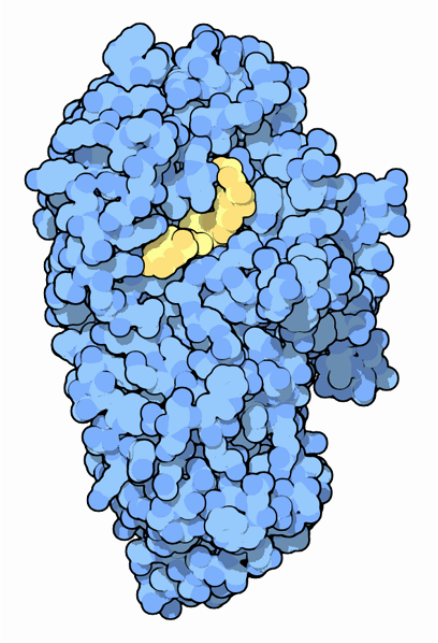
6. Click **'By Title'** and browse alphabetically to **'Alpha-Amylase'**, or type **'Alpha-Amylase'** in the search bar.

   Read the information about this enzyme and describe it in detail:

   - *Where in the human body α-amylase is found.*
   - *The natural substrate of this enzyme.*
   - *The catalytic function of α-amylase.*

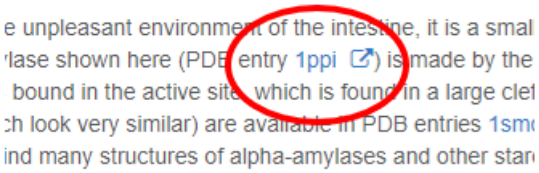
   What is the yellow part in the image on the right?

Structure of α-amylase from the pig pancreas (PDB ID: 1PPI)



Visualizing the protein structure in the PDB database:

7. Click on the PDB entry 1PPI from the Molecule of the Month page about α-amylase.



8. Click **"Structure."**

Representation of Protein Structures

Proteins can be described at different structural levels:

- Primary structure – the amino acid sequence.
- Secondary structure – organized regions of the protein (α-helices and β-sheets).
- Tertiary structure – the complete 3D structure of a single protein chain.
- Quaternary structure – some proteins consist of multiple chains.

Proteins are very large molecules, and there are different ways to represent their structure:

- Cartoon
- Ball-and-stick
- Space-filling

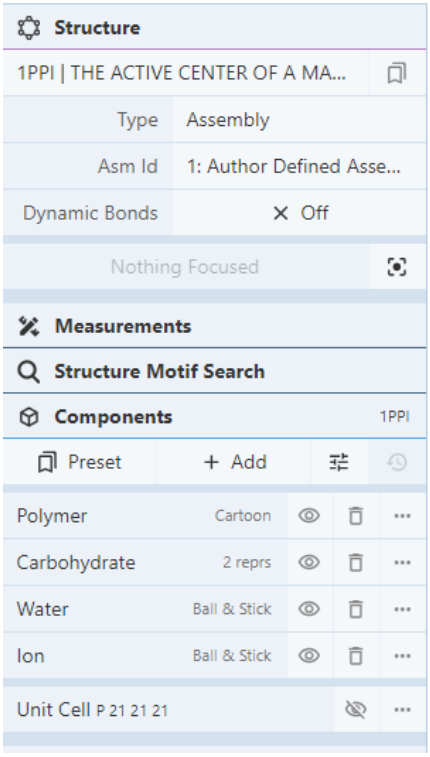
Cartoon Representation

In this model, the protein's main chain is visualized in different ways:

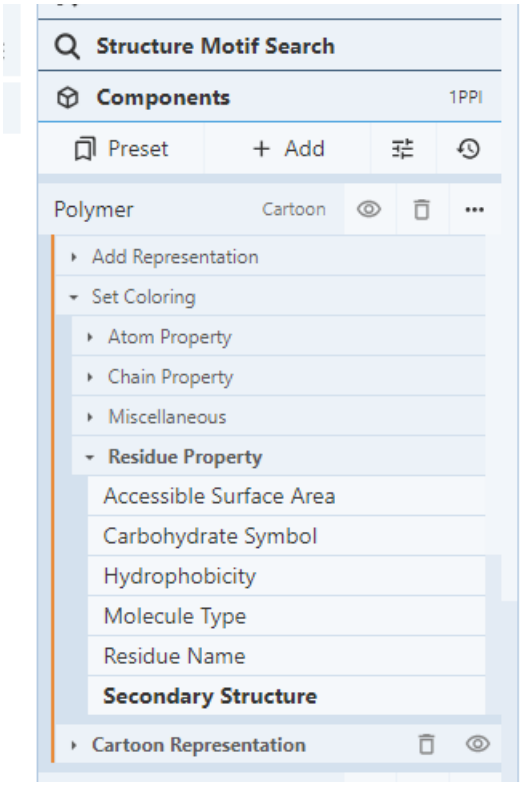
- α-helices appear as coils.
- β-strands are shown as arrows.
- Unstructured regions are displayed as chains.

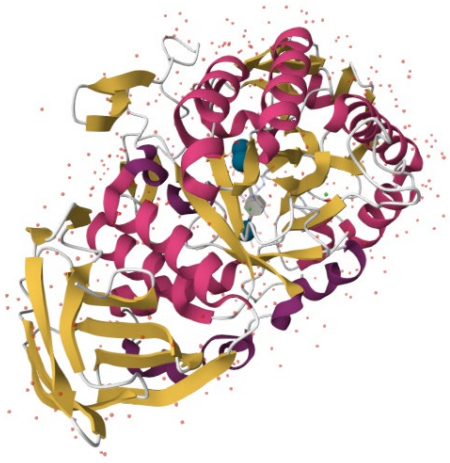
In the PDB viewer, the default representation is the cartoon model.

On the right side of the screen, you will find a menu.



9. Click on **Polymer (...)** → **Set Coloring** → **Residue Property** → **Secondary Structure**.



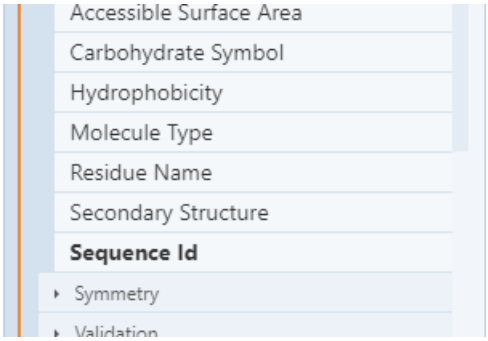


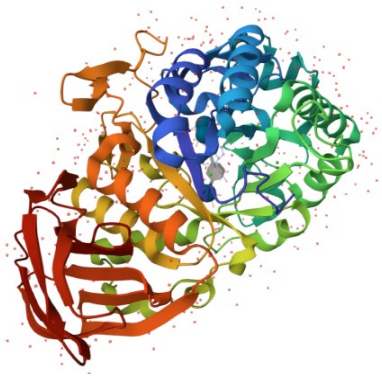
Using different colors, we can identify the two secondary structures, α-helices and β-strands:

- **α-helices** – pink/purple
- **β-strands** – yellow
- **Other parts** – white

How many α-helices can you find in α-amylase?

10. Click on **Polymer (...)** → **Set Coloring** → **Residue Property** → **Sequence ID**.





The coloring of the chain transitions from blue to red, resembling a rainbow, corresponding to the N-terminus and C-terminus.

11. Search for the N-terminus and C-terminus of the protein. When you hover the cursor over the terminus, you can read the name of the amino acid in the bottom right corner.

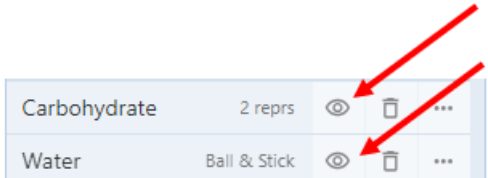
What amino acid is located at the C-terminus?
What amino acid is located at the N-terminus?

12. Rotate the protein by clicking the left mouse button. Rotating the protein structure allows you to observe how the secondary structures form unique conformations. The β-strands of α-amylase form a cylindrical structure called a β-barrel.

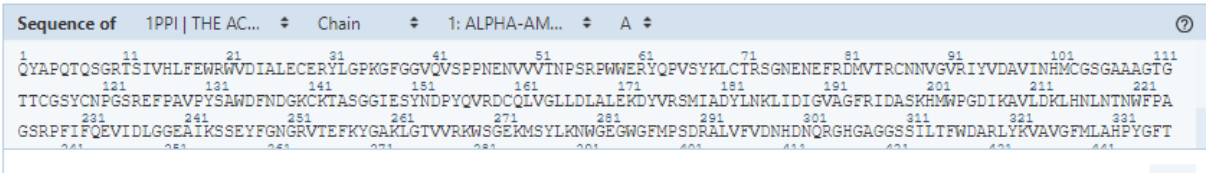
In this study, a substrate is bound to the α-amylase protein.

13. You can make the substrate (in)visible by clicking the eye icon. Click the eye icon next to **Carbohydrate**.

The dots around the protein represent water molecules. Click the eye icon next to **Water** to make these molecules invisible.

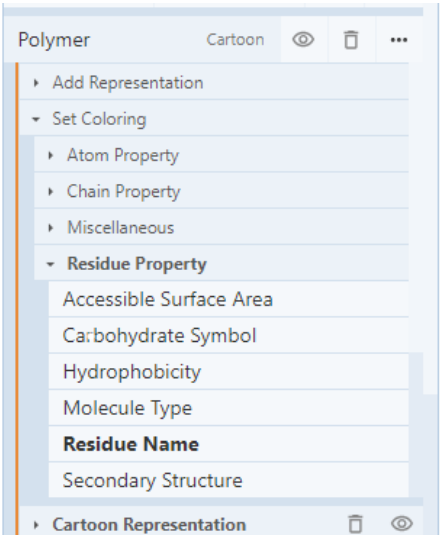


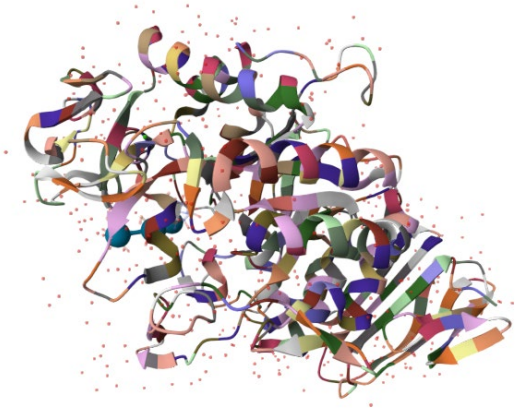
The amino acid sequence of the protein can be found at the top of the screen.



How many amino acids are in the α-amylase chain?

14. To color each amino acid in different colors, click on
Polymer (...) → Set Coloring → Residue Property → Residue Name.





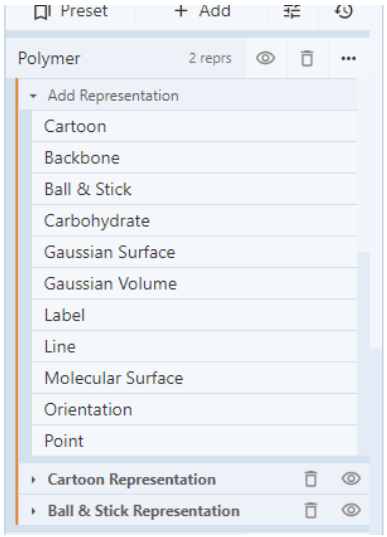
Ball & Stick Model

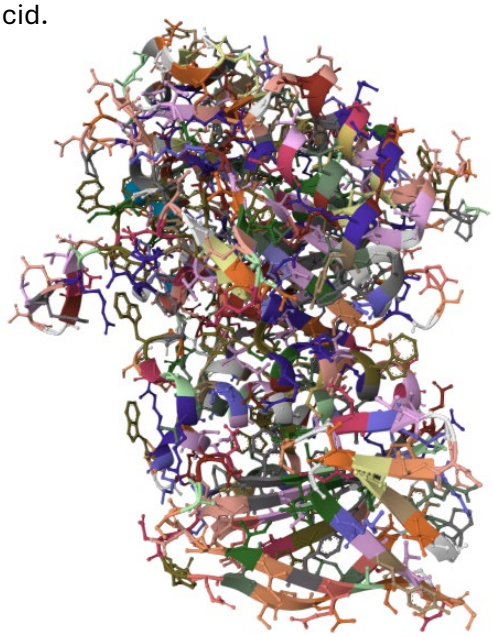
So far, in the cartoon representation, we have studied the main chain of the protein. This model makes it easy to observe the secondary structures of the protein to visualize the folding of the protein.

To complete the structure, the protein also consists of side chains that are attached to the main chain.

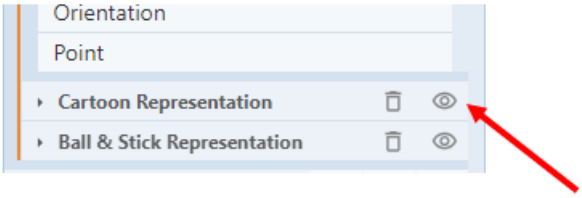
15. To visualize the side chains, click on:
Polymer (...) → Add Representation → Ball & Stick

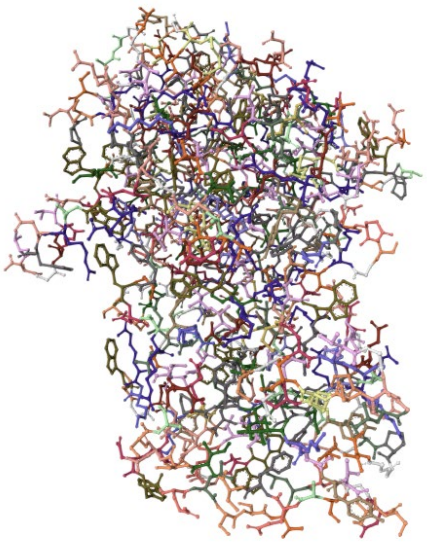
This will allow you to see the side chains of each amino acid.





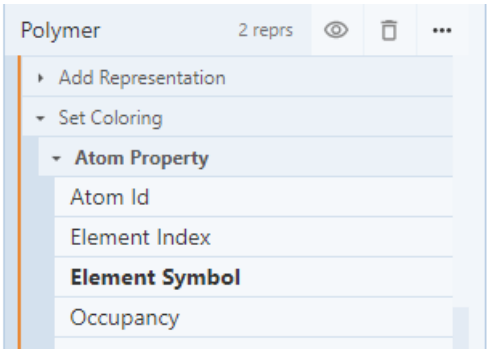
16. The main chain is still shown in the cartoon representation. To make this representation invisible, click the eye icon next to **Cartoon Representation**.

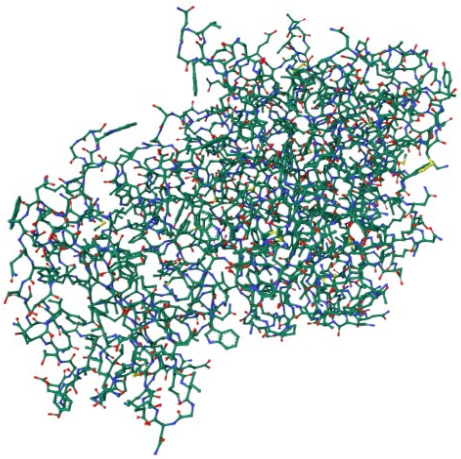




Now, the complete protein is displayed in a ball-and-stick model. Each color represents a specific amino acid. Each atom is represented as a ball, and the bonds between atoms are shown as sticks. You can also assign a color to each atom.

17. Click on **Polymer (...)** → **Set Coloring** → **Atom Property** → **Element Symbol**.



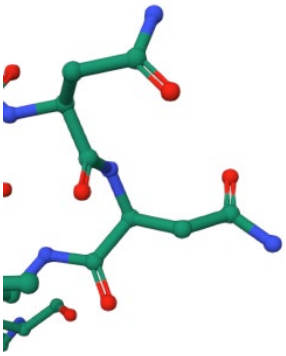


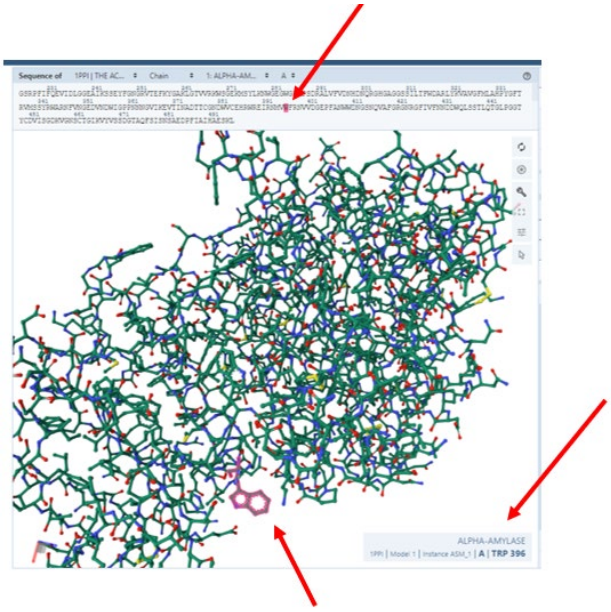
Carbon – green

Oxygen – red

Nitrogen- blue

Sulfur – yellow





Hydrogen atoms are not visible in this representation, but it's easy to identify the disulfide bonds.

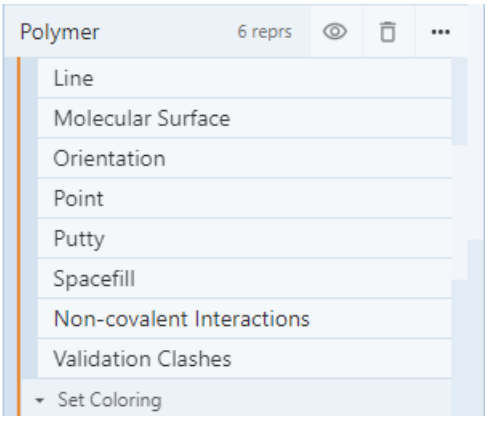
How many disulfide bonds can you find in α-amylase?

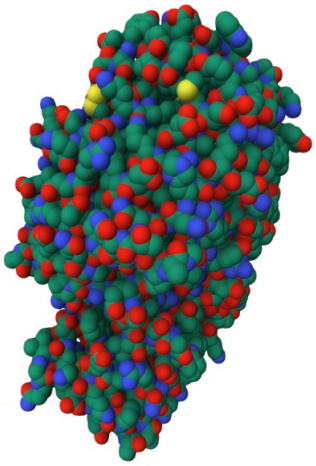
When you hover the cursor over an amino acid, the name of the amino acid will appear in the bottom right corner. At the top of the page, you'll see that particular amino acid highlighted in pink along the chain.

Space-Filling Model

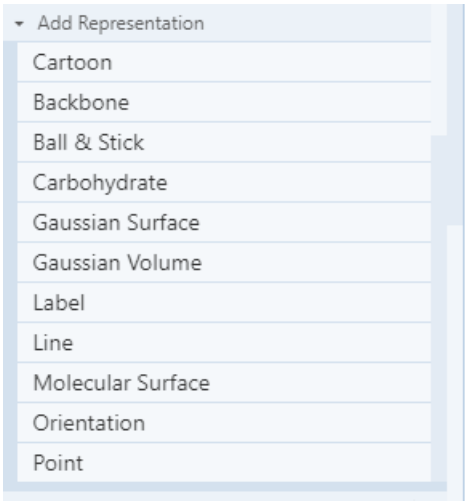
In this model, the atoms are represented by spheres, with their size proportional to the actual size of the atoms.

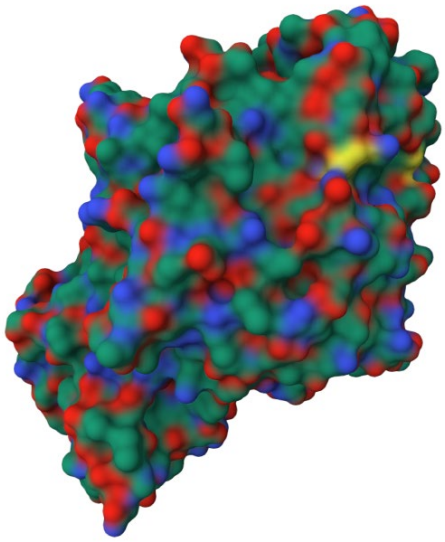
18. Click on **Polymer (...) → Add Representation → Spacefill.**



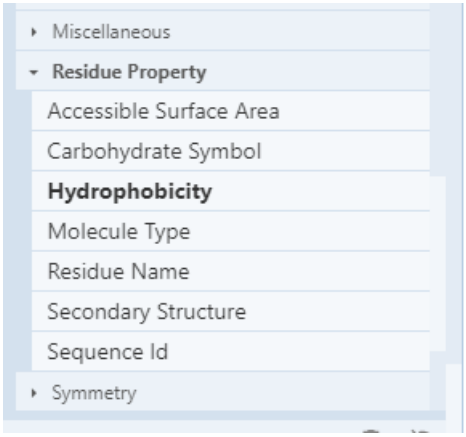


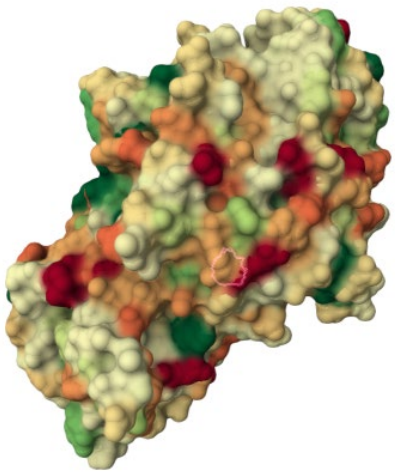
19. Click on **Polymer (...)** → **Add Representation** → **Molecular Surface** to visualize the outer molecular surface of the protein.





20. Click on **Polymer (...)** → **Set Coloring** → **Residue Property** → **Hydrophobicity** to display the hydrophilic and hydrophobic regions of the protein.





Hydrophilic amino acids are colored red/orange, while hydrophobic amino acids are colored green.

Which of the representations (cartoon, ball & stick, or space-filling) is the most accurate representation of reality?

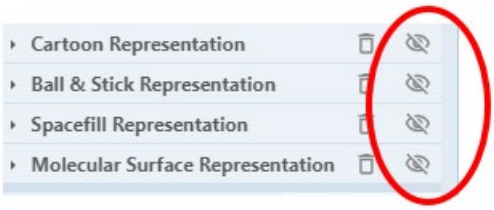
What is the disadvantage of this model?

The Substrate

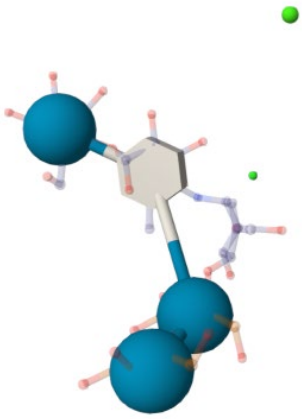
Recap:

- *What is the natural substrate of this enzyme?*
- *What is the catalytic function of α-amylase?*

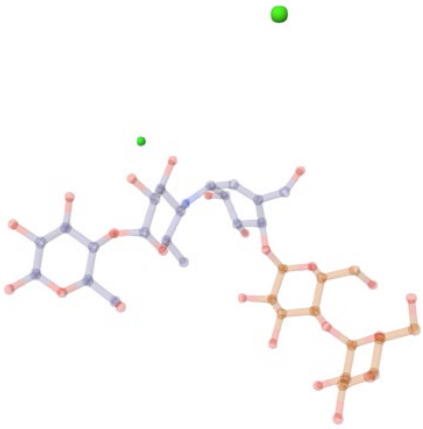
In this study, a substrate is bound to α-amylase. The substrate is a carbohydrate. To focus on the substrate in more detail, we make the protein invisible by clicking the eye icon next to each representation of the α-amylase protein.

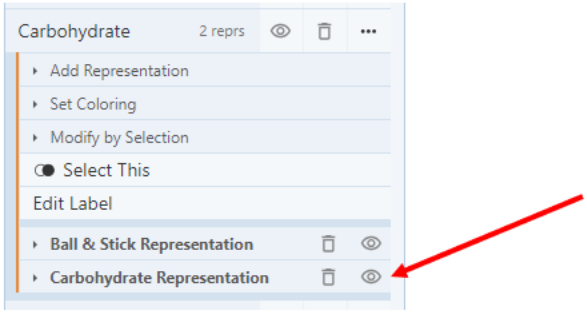


Now, only the carbohydrate and two ions (a calcium ion and a chloride ion) are visible.



The substrate is partly represented as a carbohydrate model and partly in a ball-and-stick model. We can make the carbohydrate representation invisible by clicking the eye icon.

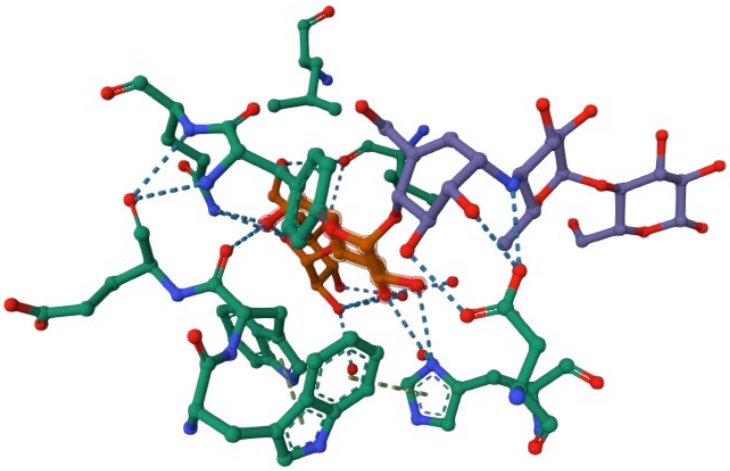




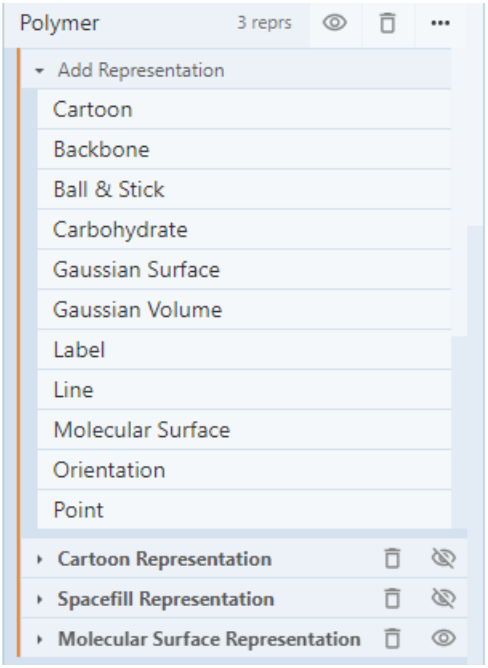
Now, it's easy to see that the substrate consists of five carbohydrate units.

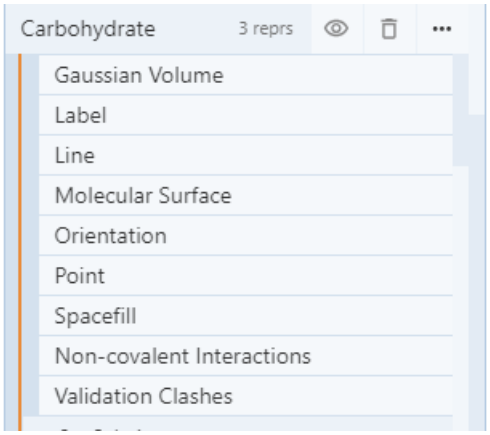
21. After switching to the ball-and-stick model for both the α-amylase protein and the carbohydrate substrate, click on the substrate. This makes it easy to identify the hydrogen bonds between the active site of the protein and the substrate.

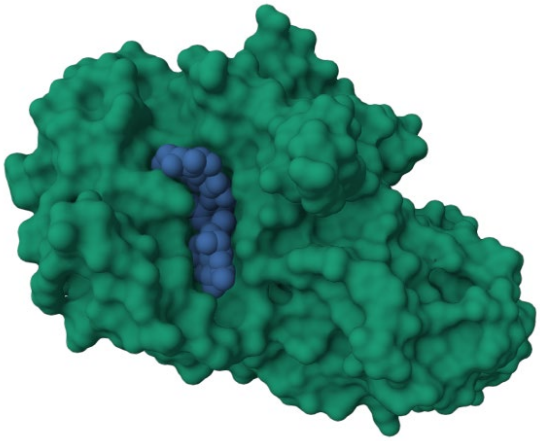
22. Name the amino acids involved in this binding.



Using the space-filling representation for the substrate (**Set Coloring** → **Atom Representation** → **Element Index**) and the molecular surface for the protein, it is shown how the carbohydrate substrate fits like a key in a lock within the active site of the α-amylase protein.







Activity 3: The Anfinsen Experiment

1. Overview

In this activity, students are introduced to the Anfinsen experiment, which demonstrated that a protein's amino acid sequence determines its three-dimensional structure.

2. Learning Goals

Students learn how Anfinsen's experiment supports the idea that a protein's amino acid sequence determines its three-dimensional structure.

3. Assessed Outcome

Students will gain an understanding of the self-assembly process of protein folding and Anfinsen's pioneering research on this topic.

4. Key Vocabulary

Protein Folding, Anfinsen's experiment, Ribonuclease A

5. Materials & LabXchange Pathway(s)

Computers
Worksheet

6. Teacher Preparation

If students need extra support, the teacher should prepare their own resources to teach Anfinsen's experiment.

Share the document **"The Anfinsen Experiment"**.

7. Lab Safety Considerations

Not applicable.

8. Sequence of Activities

Activity Description	*Time*	*Materials*
Students make summary notes from the resource and address the questions. Students discuss their findings in small groups to check their understanding.	90 min	Document- The Anfinsen Experiment

The Anfinsen Experiment

Students read through Chapter 2.1 from the Protein Folding reader and/or watch the video from the AKLextures website.

Students make summary notes from the resource and address the questions. Students discuss their findings in small groups to check understanding.

If access is permitted via your institute log-in system:
Download the pdf version of Protein Folding, An Introduction, Gomes, C.M., Faisca, P.F.N.(2019) DOI:10.1007/978-3-319-00882-0_1. https://link.springer.com/chapter/10.1007/978-3-319-00882-0_1
Read Chapter 2.1 The Anfinsen Experiments (pages 11 to 13), especially the text explaining Figure 6.

If access is not permitted, use the AKLecture about Anfinsen's Experiment of Protein Folding.

Have students answer the following questions:

- Why did Anfinsen choose Ribonuclease A for the protein folding study?
- Describe the Ribonuclease A protein in detail
- What is the substrate of Ribonuclease A?
- What reaction is catalyzed by Ribonuclease A?
- Provide a general description of the structure of Ribonuclease A. (PDB ID 1AFU)

Consider the following questions:

a. Is it a monomer or multimer protein?
b. Does it consist mainly of α-helices or β-strands?
c. How many disulfide bonds do you count?

- What agent used Anfinsen to break the covalent disulfide bonds?
- What agent used Anfinsen to break the non-covalent interactions (e.g. hydrogen bonds and ionic interactions)?
- Watch the video dialysis and explain how dialysis can remove the two agents.

Finally, explain each step of Anfinsen's experiment to each other in small groups.

Activity 4: A Protein Folding Experiment

1. Overview

Following in the footsteps of Anfinsen, students will investigate the unfolding and refolding of a protein to study the self-assembly process, in which a protein sequence spontaneously forms a unique native state. In this experiment, α-amylase will be unfolded using guanidine chloride. After removing this protein denaturant, students will investigate the refolding of the protein by measuring the enzyme activity of α-amylase.

2. Learning Goals

Students will:

- Develop general research skills, such as:
  - Organization and planning
  - Communication and cooperation
  - Using experimental controls
  - Concluding data
- Develop lab techniques, such as:
  - Unfolding proteins using denaturing agents
  - Separating molecules in mixtures using gel filtration
  - Detecting proteins in fractions using the Bradford protein assay
  - Detecting enzyme activity of α-amylase using iodine solution to test for the presence of starch
- Learn that proteins have the self-assembly capacity to fold into an active native state.

3. Assessed Outcome

Students will investigate protein folding experimentally using technical lab skills such as gel filtration, protein assay, and α-amylase enzyme activity assay.

4. Key Vocabulary

Protein folding, denaturing agents, gel filtration, protein assay, α-amylase enzyme activity assay.

5. Materials and LabXchange Pathway(s)

Lab equipment as described in the protocol for the experiment.

6. Teacher Preparation

Share the document **"The Art of Protein Folding - Experiment"**.

7. Lab Safety Considerations

- Handle all reagents with care, following proper lab protocols.
- Dispose of all chemical waste according to your institution's guidelines.

8. Sequence of Activities

Activity Description	*Time*	*Materials*
1. Experiment Parts 1 and 2 Gel Filtration – Separation of Two Dyes	90 min	Document - The Art of Protein Folding - Experiment
2. Experiment Parts 3, 4, and 5 Unfolding and Refolding of α-Amylase	90 min	
3. Poster Presentation A detailed study of the laboratory techniques used in the experiment.	90 min	

The Art of Protein Folding - Experiment

This experiment was designed in collaboration with Prof. Dr. S.G.D. Rüdiger from the Faculty of Science, Chemistry, Cellular Protein Chemistry, Utrecht University, as part of a project 'Teacher at a Department' at Utrecht University.

Lesson 1: Gel Filtration – Separating Large and Small Molecules

Part 1 – Separation of Two Dyes (Experimental Activity)

Equipment:

- PBS (phosphate-buffered saline, pH = 7.2, in physiological salt)
- PD-10 column stored in PBS
- A mixture of Phenol Red and Dextran Blue in PBS
- 10 empty microtubes

Figure 1
PD10 column held by a clip

You are provided with a mixture of two dyes. One dye consists of large molecules, while the other is very small.

The PD-10 gel-filtration column is a plastic tube filled with a gel-like substance, with a layer of water on top. A clip secures it.

1. Carefully examine the tube.
   - ***a.*** *Do you notice a filter at the bottom that holds the gel inside the column?*
2. Another filter is placed on top of the gel.
3. Remove the cap from the top and bottom of the column.
   - ***a.*** *What do you observe?*
4. Collect the liquid in the beaker below until no liquid remains at the top of the column.
5. Replace the cap at the bottom.
6. Apply 5 drops of the dye mixture on top of the gel.
7. Remove the cap at the bottom and allow the dyes to soak into the gel.
8. Collect the eluate in a microtube placed beneath the gel column.
9. Carefully apply a few drops of water to the gel and collect the eluate in a microtube underneath the gel column.
10. Repeat this step several times by adding 1 mL of water to the gel and collecting the eluate in a new microtube each time.
    - ***a.*** *Which dye is eluted first from the column: the red dye or the blue dye?*
    - ***b.*** *How many mL of water are needed to elute the blue dye?*
    - ***c.*** *How many mL of water are needed to elute the red dye?*

Part 2 – Separation of Two Dyes (Theoretical Activity)

Gel filtration is a separation technique based on differences in molecular size.
The gel consists of small spheres with tiny pores. A model of such a sphere is shown below

Figure 2: Transverse section of a PD-10 gel sphere, showing some pores.

The two dyes in this theoretical experiment differ significantly in molecular size.
The green dye consists of large molecules, while the purple dye consists of small molecules.

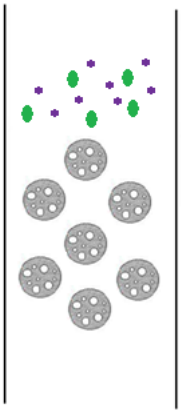


Figure 3: Model of a PD-10 column, with a mixture of two dyes applied to the top. The green dots represent large molecules, while the purple dots represent small molecules.

a. *When we apply a mixture of large (green) and small (purple) molecules to the gel, which molecules can enter the gel's pores, and which cannot?*

In the figure above, a model of a tube filled with gel spheres is shown. Draw the path that the large molecules (green) would take through the gel.

b. *Do the same for the small molecules (purple).*

c. *Which molecules will travel a longer distance in the gel—the large or the small ones? Which of the two dyes will elute first from the column?*

d. *Referring back to the experiment in Part 1: which molecules are larger, the red dye molecules or the blue dye molecules?*

e. *Measure the dimensions of the gel column (diameter and height). Calculate the volume of the gel in the tube.*

f. *Comparing the answers to questions Part 1 c and d with the total volume of the gel, what conclusions can you draw?*

g. *Explain why the blue dye traveled over about 2 mL, while the red dye traveled over more than 5 mL.*

h. *If you have a mixture of two dyes, both with large molecular sizes, how could you use gel filtration to separate these dyes?*

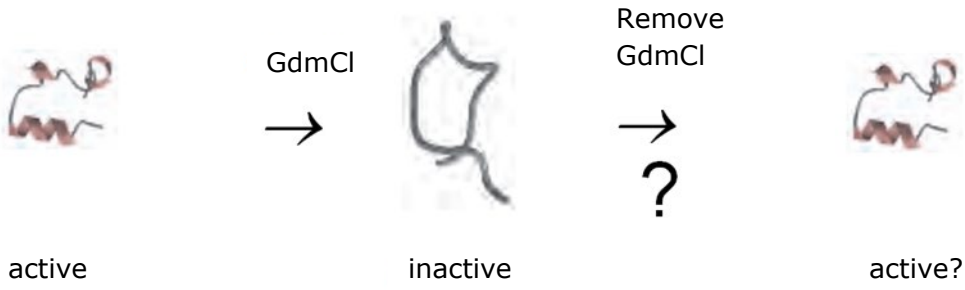
Lesson 2: Unfolding and Refolding of α-Amylase

Let's explore the study of (un)folding the protein α-amylase.

Proteins fold into a three-dimensional shape that determines their activity. But how does a protein find its active form? Does it receive help from substances within cells, or can it independently find its active form in a test tube without any cellular assistance?

In this experiment, first the protein is unfolded using guanidinium chloride (GdmCl), a strong denaturant. At high concentrations of GdmCl proteins lose their ordered structure. You examine its effect on the activity of α-amylase (Part 3).

After removing the GdmCl through gel filtration (Part 4), you investigate whether the α-amylase protein can refold into its original (active) form (Part 5).



Part 3 – Unfolding the α-Amylase Protein with Guanidinium Chloride (GdmCl)

Equipment:
PBS (phosphate-buffered saline, pH = 7.2 in physiological salt)
Iodine solution
Bradford reagent

Microtube A (active enzyme): 0.5 mL of 2% α-amylase in PBS
Microtube B (inactive enzyme): 0.8 g of GdmCl added to 0.5 mL of 2% α-amylase in PBS (prepare just before use)
Note: In this activity, α-amylase is already unfolded by GdmCl, but if time permits, students can prepare microtube B by adding GdmCl to the microtube.
Microtube Z: 1.5 mL of 1% starch solution

Plastic cards 1 and 2
PD-10 column stored in PBS
10 empty microtubes

The Effect of GdmCl on the Activity of α-Amylase

a. *Why do we need to dissolve the enzyme α-amylase in PBS?*

Measure the α-amylase activity in microtubes A and B by adding a drop of iodine solution to a drop of 1% starch in PBS on plastic card 1, in squares A, B, and C, according to the scheme below.

A	B	C	
1 drop of 1% starch 1 drop of 2,5 % α-amylase (microtube A) After a minimum of 5 minutes, add: 1 drop of iodine solution	1 drop of 1% starch 1 drop of 2,5 % α-amylase **+ GdmCl** (microtube B) After a minimum of 5 minutes, add: 1 drop of iodine solution	1 drop of 1% starch 1 drop of PBS After a minimum of 5 minutes, add: 1 drop of iodine solution	

b. *Why do we need to test PBS (C) as well?*

c. *Explain your observation. Use terms such as enzyme activity and unfolding in your answer.*

The enzyme activity of α-amylase, which breaks down starch into glucose, can be measured using an iodine (I_2) solution. Iodine causes starch to turn blue, while glucose does not react with iodine. Therefore, if starch is broken down into glucose by α-amylase, no blue color will appear after adding iodine.

Starch + I_2 (yellow) → starch-I_2 complex (blue)
Glucose + I_2 (yellow) → no complex formation

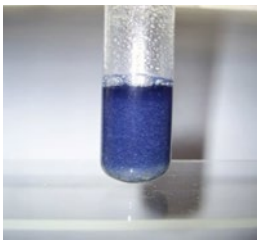


Figure 4:

Starch with I_2 turns blue

Part 4 – Self-Assembly in the Folding of the α-Amylase Protein

4.1 Removing Guanidinium Chloride (GdmCl) Using Gel Filtration

To remove GdmCl from the protein solution, we will use PD-10 columns.
In our introductory experiment, where we separated a mixture of two dyes, we learned that gel filtration separates large molecules from small ones. In this experiment, we will separate the α-amylase protein and GdmCl in the same way we separated the dyes.

1. Place a cap at the bottom of the PD-10 column.
2. Apply 10 drops of the α-amylase solution containing GdmCl (from microtube B) to the top of the column.
3. To allow the protein to enter the column, release the cap at the bottom.
4. Add 1 mL of PBS repeatedly, collecting 1 mL each time, to gradually flush both the protein and GdmCl through the column. Continue collecting the solution in a microtube, 1 mL at a time, until you have added and collected 10 mL in total.
5. Afterward, rinse the column with 15 mL of PBS and collect the resulting droplets in a beaker.

4.2 Detecting the Presence of Protein in the Fractions Using the Bradford Assay

Since proteins and GdmCl are colorless (unlike the dyes used in the initial experiments), we need an assay to detect which fraction contains the eluted protein. To identify the presence of proteins, we will use the Bradford reagent.

a. *Based on the results from the introductory experiment with the dyes, after how many mL do you expect the α-amylase protein and GdmCl to elute from the column?*

The Bradford protein assay is a simple, fast, and accurate method for detecting proteins. The dye Coomassie Brilliant Blue changes color in the presence of protein.

Use the Bradford reagent to investigate whether protein is present in the fractions collected in the 10 microtubes. Transfer 4 drops from each microtube onto plastic card 2 and add 1 drop of Bradford reagent, following the diagram on the card.

1	2	3	4	5
6	7	8	9	10
11	12	13		

Square 1 to 10: 4 drops from microtube 1 to 10
In square 11: 4 drops from microtube A
In square 12: 4 drops from microtube B
In square 13: 4 drops of PBS
+
in each square 1 drop of Bradford reagent

b. *Which test do you perform in squares 11, 12, and 13? What color does the reagent turn in the presence of protein, and what color does it turn in the absence of protein? What effect does GdmCl have on the Bradford Protein Assay?*

c. *By inspecting the color of fractions 1 to 10, how many mL were needed to elute the α-amylase protein? Does this match your expectations?*

d. *How many mL do you expect to need to elute GdmCl?*

e. *How could you test whether the α-amylase protein has regained its original functional form? Design an experimental plan, discuss it with your supervisor, and perform the experiment in the empty square on plastic card 1.*

To evaluate, students communicate the results with the teacher. Optionally students write a report of their experimental process and results.

Lesson 3 Background Information

In the final task of this project, students will work together in small groups, allowing them to learn from and teach each other.
In a 'home' group of four or five students, each member will search for background information on the internet about the following topics:

- Gel filtration (Sephadex, PD-10 columns)
- Bradford Protein Assay (Coomassie Brilliant Blue)
- The α-amylase activity assay (Starch and Iodine)
- Comparing the Anfinsen experiment on protein folding with the α-amylase (un)folding experiment
- α-amylase

Each student in a group is assigned a topic and independently deepens their knowledge of it. They become the "expert" in their group in this specific area.

In the next step, students with the same topic from different groups come together to share their knowledge and collaboratively come to high-quality answers. They prepare a poster presentation and return to their 'home' group. Finally, each student will present their topic to their 'home' group.

Possible questions to address in each topic:

1. **Gel Filtration (Sephadex, PD-10 columns):**
   How does gel filtration work, and what is the principle behind the separation of molecules based on size?
   - How do the pore size and the type of gel material affect the separation of molecules during gel filtration?
   - What are the advantages of using PD-10 columns for purifying proteins or other biomolecules?
   - Why is gel filtration useful for removing impurities such as salts or solvents from protein samples?

2. **Bradford Protein Assay (Coomassie Brilliant Blue):**
   What is the mechanism behind the Bradford Protein Assay?
   - How does Coomassie Brilliant Blue change color in the presence of proteins, and why does this happen?
   - What are the limitations of the Bradford Assay compared to other protein assays such as the BCA assay or Lowry assay?
   - What factors can affect the results of the Bradford assay, such as the presence of other substances in the sample?

3. **The α-amylase activity assay (Starch and Iodine):**
   How does the α-amylase activity assay with starch and iodine work?
   - Why does the solution color change when α-amylase breaks down starch? What happens at molecular level?
   - How can you quantify α-amylase activity using iodine solution?

4. **Comparing the Anfinsen Experiment on Protein Folding with the α-amylase (Un)folding Experiment:**
   How do the Anfinsen experiments relate to the (un)folding of α-amylase experiment?
   - How does the Anfinsen experiment support the idea that the amino acid sequence of a protein determines its three-dimensional structure?

- What are the similarities and differences between the experiment of renaturing α-amylase after denaturation with GdmCl and Anfinsen's ribonuclease experiment?
- What role do environmental factors, such as ions or pH, play in restoring α-amylase activity after denaturation?
- What can the Anfinsen and α-amylase experiments teach us about protein self-folding in cells versus in a test tube?

5. **α-amylase:**
   - In which organisms can amylase be found?
   - Where in the human body can you find α-amylase?
   - What is the substrate of α-amylase? (Look for a picture of the chemical structure of the substrate)
   - What reaction is catalyzed by α-amylase?
   - At which pH is salivary α-amylase most active?
   - How does α-amylase differ from other forms of amylase?
   - Provide a general description of the structure of α-amylase. Consider the following points:
     a. Is it a monomer or multimer protein?
     b. Does it consist mainly of α-helices or β-strands?
     c. Does α-amylase have bound co-factors like metal ions?
   - Are calcium or chloride directly involved in the enzymatic reaction? If not, what can you say about their function?
   - Can you predict where the sugar binds? Describe where you think the binding site is.
   - What can you say about the properties of the surface where the substrate of α-amylase, starch, should bind?

