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SARAH HEWETT: Today, we are going to talk about the second part of the catalase lab. So, in the first part of the catalase, we had catalase and hydrogen peroxide. And we were measuring the rate of the decomposition of hydrogen peroxide as catalyzed by the catalase enzyme. And we were doing that by measuring the pressure of oxygen.

And, for the people who are currently doing the catalase lab, you've already done part one. And, for everybody else, you'll get to experience that coming up in the next lab or two. And then, in part two of the catalase lab, we are going to look at quantifying the amount of catalase that we have in a sample and then looking at the iron centers in the catalase and trying to quantify those as well.

So we are going to go back to the beginning and talk a little bit more about proteins and protein structure. So, if you have taken a biology course at any point in your life, then this will probably be review. But, just so that we're all on the same page, we want to go back and say that the building blocks of proteins are amino acids. And this is the general structure of an amino acid. And, if you change this R group here to any one of these R groups over in this table, then you can get all of the different amino acids that are naturally produced in nature.

And there are 20 amino acids that occur in nature. And they vary by these R groups. And, depending on the R group, there are different classes of amino acids that you can have. So there are the nonpolar ones where you have an alkyl or an aromatic side chain. You can have polar side groups where you have a hydroxyl group or a thiol or some amine groups.

And then you can have acidic or basic groups that are electrically charged. So, depending on the pH of the solution, they'll either be protonated or not. And then you can have positive and negative charges. And all of those contribute to the protein structure and the properties of the protein, what it can do.

And just a review of terminology, so proteins are made of chains of amino acids. A peptide is two or more amino acids stuck together. A polypeptide is when you get 10 or more amino acids stuck together. And then, if you have beyond that, once it gets to longer and longer chains of amino acids and they start folding up and having specific functions, that's when it becomes a protein. So, if you hear those terms, that's what we're talking about.

So the first-- the most basic structure that you can have in a protein is the primary structure. And that is just the sequence of amino acids. So you have this long chain of amino acids. You stick them together. And that is the primary structure.

So, if this is our little peptide chain here, we have alanine, threonine, tyrosine, and valine. And those are from these side groups is how you know what amino acid it is. And then the bond in between the amino acids here where your carbonyl group of one amino acid bonds to the amine group of another amino acid, that is your peptide bond. And so you can get chains of these, and the primary structure again is just the list of amino acids in a line. That's about as simple as it gets.

The secondary structure is how the amino acids interact with one another to form 3D structures within the polypeptide chain. So, if you have two chains of amino acids or if you have one that's folded onto itself, it can form hydrogen bonds between these oxygens of the carbonyls and the hydrogens that are attached to the nitrogens in the protein backbone. And you can get these beta sheets that are kind of pleated if you look at them in actual 3D space.

And you can have one long chain that bonds to itself in the similar fashion with the hydrogen bonding. And you can get alpha helices. And it creates a helical shape within the protein backbone.

And these are the main types of secondary structures that you can get. There are other ways that the protein can interact with itself and form different structures, but these are the major two. Structures are held together using hydrogen bonds.

And then you can get tertiary structure. So there are many different ways that proteins are represented in graphical form. So you can have-- you've seen in our past lecture with the catalase, you can have a space-filling model where all of it's kind of like the ball and stick where it just looks like one giant blob. And that can give you a sense of the overall shape of the protein.

You can also have line structures where it's kind of like the line structure that you would draw in organic chemistry where it shows all of the different side chains in the backbone. And then you have these ribbon structures where you can see sort of the alpha helices and maybe some beta sheets over here. And it shows some different features of the structure of a protein.

But the tertiary structure is how all of the elements of the secondary structure interact to form the overall shape of the protein. And it can be formed by various interactions. And so, while the secondary structure is mostly formed by interactions between the backbone functional groups, the, tertiary structure comes from the interactions between the side chains. So you can have these hydrophobic interactions if you have alkyl or aromatic side chains.

So, if your protein is in a cell, then it is in a mostly aqueous environment. And aqueous environments are polar. And so these nonpolar groups will tend to coagulate together.

You can have disulfide bonds. So, if you have the protein side chains that have sulfur groups, then they can make covalent bonds between the sulfurs. You can have hydrogen bonding. Again, those different side chains have again hydrogens that are attached to nitrogens and oxygens and carbonyl groups that can form hydrogen bonds.

And then those electrically charged side chains can form salt bridges. So, if you have a positively charged and a negatively charged amino acid near each other, then they can have an ionic interaction like that. Interactions with the solvent also play a role. And this is what holds the protein together in its functional shape.

And then, lastly, we have our quaternary structure. And this is formed by the interaction of multiple subunits coming together to make a larger protein. So this is a picture of catalase. And you can see each of the subunits of the catalase is a different color.

So catalase has four different subunits. So these four subunits all have their own secondary and tertiary structure. They're identical. And then they come together to make the overall catalase protein.

Not all proteins have quaternary structure. Some just have normal folding and secondary and tertiary structure. And the quaternary structures are typically held together by dispersion forces and hydrogen bonding between the subunits. So it's not usually a covalent interaction, but it is strong enough to hold these subunits together into the correct shape for the protein to function.

Another key piece of protein structure are prosthetic groups. And these are groups that are not made out of amino acids. So they are not peptides, but they are very important for protein function. And some examples are iron-sulfur clusters. So these are some 3D representations of what an iron-sulfur cluster would look like where your iron centers are red, and the sulfur are these yellow balls.

So you can have different numbers of irons and sulfurs bonded together. And these play key roles in photosynthesis and in metabolism. So they're really good at electron transport. So, in the electron transport chain in your cellular metabolism or in photosynthesis when you have to transport electrons in order to make energy, those iron-sulfur clusters, that is where they are typically found in nature.

And another really common prosthetic group is the heme group, which you may recognize from hemoglobin. It is used in oxygen binding and transport. So it binds to the oxygen and carries it through your blood. And it can also be used in oxygen reactions or oxygen activation, which is what we're going to talk about today. Other types of prosthetic groups, you can have lipids or sugars that are bound to proteins that help with protein recognition and things like that, but metals and metals centers are the most common that are used.

So, if we want to talk a little bit more about the heme group is what we're going to focus on, the heme group is based off of this porphyrin structure. And so this is a porphyrin. It is made up of different pyrrole rings that are bridged together. And, if you look at this shape, there's a nice hole in the center, which is perfect for inserting a metal center.

And pyrroles are found-- or porphyrins are found in many different contexts. So you can find them in hemes. Obviously, we've said that already. So, if you put an iron center in the middle of that and you can add different functional groups to the outside of the pyrrole, then you can make a heme.

In chlorophyll, it has a magnesium ion in the center of it. Enzymes have these to hold a whole bunch of different metal centers or even to not hold any metal centers, just the very common organic group.

The organic chemists said, wow, these enzymes use all of these porphyrin groups, and it does some really interesting redox chemistry. So we can make these in the lab. So they have made synthetic versions of porphyrins with manganese, iron, and cobalt that catalyze a bunch of reactions in the lab, not necessarily in nature.

And they're also found in petroleum complexed with vanadium and nickel. And one of the ways that they're used in the petroleum industry is to fingerprint petroleum. So, by analyzing the composition of the porphyrin complexes in the petroleum, they can detect the source of oil spills. They use it in geochemistry and forensics and things like that.

So now, if we look back at our heme molecule, you can see that all of the hemes contain this porphyrin structure. And then you can add different groups around the outside, and that will change the structure. And that will change the function and change the way that it is able to bind to different proteins.

So some of the hemes are just kind of stuck and held together with non-covalent interactions. And some hemes can actually be covalently bound to proteins, depending on how they are functionalized. And, like we said, they're good for oxygen binding, oxygen transport, and oxygen activation.

And one of the interesting things that I found was the role of hemes in Impossible meat. So have you guys heard of the Impossible meat or the Impossible Burgers, the Impossible Whopper? Has anybody tried it? Yeah? What are-- is it actually like meat? What do we think? Is it good? I haven't had it personally.

AUDIENCE: It's pretty good.

SARAH HEWETT: Pretty good. Mixed reviews? Not so great? I haven't actually gotten the chance to try it, but an interesting thing, chemically speaking, about the Impossible meat is that, if you're going to have normal hamburger from a cow, then one of the proteins that is highly abundant in red meat is myoglobin.

And this is the structure of myoglobin. And you can see that this group here that is not part of the peptide arrangement is a heme. And that happens to be what gives red meat a lot of its flavor. So, when you cook it, it releases the heme, and the iron center provides a lot of flavor.

So what they did was they found that, in soybean roots, they have a protein called leghemoglobin, which is very similar in structure to myoglobin and also contains a heme center. And so, if they extract this protein out of the soybean plant roots, then they can add it to the Impossible meat. And then you will have a plant-based source of heme that will give you the meaty, irony flavor that we have associated with red meat.

So they take proteins from soybeans and potatoes, like they make most vegetarian protein options out of, and then add this leghemoglobin from the soybean roots. And it is a red color. So it's also a colorant.

The heme center is what gives your blood its red color. So having those conjugated pi systems in the porphyrin molecule and combined with an iron-- or a metal center will give you colors. And so it gives it the color and the flavor of actual meat. So that's an interesting role of hemes outside of your blood or catalase.

Going back to catalase, again, this is the overall structure. And it is very hard to see with these colors, but there are heme centers-- you can see them-- in each of the four subunits of catalase. And it contains also a binding site for NADPH. And this is the structure of NADPH.

And the NADPH's role is to maintain the oxidation state of the iron center. So it is pretty important that the iron center maintains its iron 3 oxidation state. It goes between iron 3 and iron 4 while it is active, but, in order for it to start the chemistry, it needs to be iron 3. And so the NADPH can donate electrons to make sure that the iron is in its active form.

Now, if we want to take a moment and look at the actual mechanism for the reaction of hydrogen peroxide with catalase, this is a very pared down version of the catalase activation site. So there is a histidine residue here. You have your iron center and your heme. And then, when hydrogen peroxide comes in, the first step is that those electrons can remove one of the hydrogens from the hydrogen peroxide. And then this oxygen will bind to your metal center.

So then you have your histidine now that is protonated. You have your oxygen bound to your metal center. And then this oxygen can take the hydrogen. And the iron center can donate some electrons here to form water and an iron-oxygen double bond. And now this is iron 4.

So iron has donated some electrons to this oxygen center, and it's actually a radical and a cation. So that's the first step. And so that's where our first hydrogen peroxide molecule comes in because, if you remember, the overall reaction that we're working with is two hydrogen peroxides goes to two waters and one oxygen.

And then the second step is, once you have this iron radical cation, there are two proposed mechanisms for how this reaction can go. And it can either be also mediated by this histidine residue where one of the hydrogens ends up on the histidine. The other one adds to the oxygen bound to the metal center. You release the oxygen, and this hydrogen comes back in. And you form your water.

Or you can kind of ignore this histidine and do two hydrogen transfers onto this oxygen bound to the iron. And then you make your water and your oxygen. And they have done isotopic labeling studies where you can make this first complex and then add in hydrogen peroxide that's been labeled with oxygen-18 isotopes. And they have found that all of the oxygen that is produced is oxygen-18.

So they know that the oxygen here comes from this peroxide. And then the oxygen that is bound to the iron ends up in the water. So that's kind of how they were able to narrow down some of the mechanisms for this.

And, if you remember, our reaction, our decomposition of hydrogen peroxide can happen on its own. So here is a mechanism for how it may happen without catalase. So you can compare. One of the proposed mechanisms is that the hydrogen peroxide breaks into two hydroxy radicals. Then the second molecule of hydrogen peroxide reacts with one of those hydroxy radicals to form one molecule of water and this peroxy radical.

And then you have one of these and one of these that are formed. So those can react and form your water and then your molecular oxygen. So it takes a few more steps. It's a little bit slower, but that is a comparison of how the enzyme can catalyze this reaction to happen much faster than what may happen in nature. So that is what is happening in the decomposition of hydrogen peroxide, either just hanging out on the bench top or with your catalase catalyzation.

And now we're going to talk about our goals for day three and four of catalase. So, after day one and day two, you have seen this reaction happen. And now, for days three and four, we are going to try to quantify the amount of catalase in an unknown protein sample. And then we were going to quantify the amount of iron that is present in our sample of catalase and try to figure out how many iron centers there are per one molecule of catalase. And, based on the work of a lot of other scientists, we know that, theoretically, it should be how many?

We can go back. So there are how many subunits in catalase? Four. How many hemes per subunit? One. So how many total iron centers in a catalase protein?

AUDIENCE: Four.

SARAH HEWETT: Four, excellent. So we know that there should be four. And hopefully-- that is our theoretical value. Hopefully, you guys are going to be able to get that as your answer in the lab.

So how are we going to do that? First, we're going to talk about how to quantify proteins. So it's important to be able to determine the amount of protein in a given sample. In terms of for nutritional studies, biochemical studies, you need to know how much protein that you have.

And there are a couple of ways that you can determine the amount of protein in a sample. You can do a nonspecific assay, which will just tell you how much overall protein that you have. It's not specific to certain proteins. And some examples of those are the biuret assay.

You can use UV spectroscopy. So we did visible spectroscopy with our phosphate samples in the Charles River lab. If you use ultraviolet light, you can detect the amount of protein that you have. So some of the amino acids have the aromatic residues or the pi bonds. And those will absorb UV light. So you can quantify proteins that way.

Or you can do a Bradford assay, which is more similar to the assay that we did with our phosphate samples in that it is a visible color change that you can see and same with the biuret assay. And the Bradford assay is actually the one that we're going to do in lab. So we'll talk about that more in a second.

And, if you want to know specifically what-- if you want to identify the amount of one specific protein, you can do protein-specific assays such as a Western blood or an ELISA. And those use antibodies to select for specific proteins. And then you can either attach a fluorescent tag to the protein or a color-changing tag. So, when the antibody matches up with your protein, you get a color change or some light that you can quantify. And you can also do protein mass spectrometry and look at-- you can use that to quantify the specific proteins also.

So the Bradford assay is the assay that we're going to be doing in the lab. And the Bradford assay uses Coomassie dye. And this is the structure of Coomassie dye. And, if you just pour the Coomassie dye out of the bottle, it is this brownish sort of reddish color. And, when it reacts to proteins, it turns blue.

So you can quantify the amount of blue. And that will tell you how much protein you have in your sample. And the protein reacts with these sulfonyl groups over here and causes the color change. So, when you have all of these aromatic systems and electron-rich groups when you change the electronic structure by bonding to a protein, then you can change the color. So this is what we are going to do.

And, in order to quantify the amount of blue or amount of protein that we have, similar to our phosphate analysis before, we need to make a series of standards. So you need to know what your absorbance is at different concentrations of the thing that you're trying to measure. So we made our phosphate standards, and those also happened to turn blue. And we are going to make a series of protein standards so that we know what color our Coomassie dye will turn a different protein concentrations.

To do this, we are going to use bovine serum albumin. And it's a serum albumin protein, which is found in blood. And bovine means that the one that we're going to be using is coming from cows. And that's helpful because there is a lot of cow blood left over from the meat industry. And it is found in the blood plasma, and it maintains-- its role in a living animal is to maintain the osmotic pressure in the blood and carry biologically important molecules through your blood plasma, but we are going to use it as a primary standard for our protein quantification.

And we can do this because it is abundant, inexpensive, stable, and reacts well with Coomassie dye. So we can get a nice calibration curve. And it's also similar enough to our protein, catalase, that we can use it to make our standard curve.

So typically you would try to use the same molecule that you are quantifying to make your standard, but that's not always possible. So, in that case, it's helpful to have a protein that can kind of stand in, is abundant, and easily quantifiable. So you can buy different concentrations of this Bovine Serum Albumin, or BSA, from a bunch of different chemical suppliers. So you know what the concentration is, and you can use that as your standard.

The way that we are going to do this in the lab is to prepare standards very similarly to how you did for your phosphate. You will have your BSA stock solution. And the BSA that we're going to be using is in solution form. And the stock solution is 2,000 micrograms per milliliter. And you will be diluting it with buffer.

So again it's really important to use buffer any time that you're working with proteins because of the different structures that we showed you before. The side chains could be charged. And that will impact their interactions with each other. So you want to make sure that the pH is correct so that your protonation states of all of your amino acids are what they should be in terms of like a physiological pH.

So you'll add-- dilute your BSA with buffer. And then you will have a bunch of different standards at varying concentrations. And then you'll have one where you don't add any BSA, and it's just the buffer. And that will serve as your blank.

The way this is going to work in lab is that you will pipette-- you'll be using little microcentrifuge tubes and the micropipetters. And so this is a very quantitative experiment. So you need to get really good with your micropipetting technique and make sure that you don't contaminate your samples because you're going to be working with very small quantities, microliters of things. So you want to make sure that you are changing your pipette tips at an appropriate time and using the pipettes accurately.

So you will pipette the standards into your microcentrifuge tubes. And then we will give you a standard sample-- or an unknown sample of catalase. And then you will put catalase in each of the microcentrifuge tubes.

And we're going to do five samples of the catalase so that we have five unknowns that you can do an analysis of your error on that, similar to the phosphate where we ran five standards from-- or five samples from the river. You guys saw how much those can fluctuate depending on how you prepared the samples or how well you pipetted. So it'll be the same thing. We will have multiples, multiple trials.

Then you will add the Coomassie reagent to each of the standards and your samples. And this is where it is going to be very important that you pay attention to the timing of this experiment. So you will add your Coomassie dye. And then you will shake it up a little bit.

And then, as soon as you add the dye, you will start a timer. And you will close the tubes, shake them, allow the color to develop for two minutes. And then, at two minutes, you will pour all of your samples into cuvettes. And, in this case, we will be using the smaller cuvettes than what you guys used for the last samples, but they go in the same instrument.

You will transfer your samples to the cuvettes. And then you will measure the absorbance at 595 nanometers. And the program on the UV/Vis instruments are set up to go to that wavelength when you click on it.

You need to start running your samples at 10 minutes. So this is why it's really important. So, once you start adding the Coomassie dye, you need to start a timer because the dye is-- it's important that all of your samples incubate for the same amount of time so that it's consistent. So you don't want to take your time like adding it to the beginning samples so that, by the time you add it to your unknowns, it's been like five minutes. So you want to work quickly and carefully.

The dye is most sensitive around 10 minutes after addition. So that's when you will get really good spectra. If you wait too long, then you will see that the protein-dye complex will start to precipitate out of your solution. And you'll get these black chunks in your cuvettes. And that's when you know that you can no longer measure the absorbance of your sample. So that'll be careful, and we will try to stagger people so that not everybody is using the UV/Vis at the same time so that you have your-- you can do everything in the allotted amount of time.

You will make a calibration curve, and it will look something like this. And, as you can see, it's not the most linear thing. BSA has three linear regions when you are doing a Bradford assay. And the first one is from 0 to 125 micrograms per milliliter. We don't really have any data points there, and your protein concentration in your catalase sample should be higher than that. So we're not really going to look at that region.

And then the other region is in the middle here, either from 125 to 1,000 or 125 to 750 micrograms per milliliter. And, when you get your data, you can look at this and graph both of these regions to see which one gives you a more linear and more steeper slope. And that's the one that you can use to calculate your unknown samples with.

And the third region is above 1,000 micrograms per milliliter, but, as you can see, you're getting above one absorbance unit there. And so then it's not as accurate beyond there. So we're not going to worry about that.

So, when you get your data, you'll need to figure out where it is most linear, where the slope is the steepest. And that is what you will use to calculate your unknown concentrations. And then it is just like you did for phosphate, and you'll be able to calculate your unknown concentrations. Any questions about the quantification of the amount of catalase protein?

So our calibration curve, also one thing to note, is in units of micrograms per milliliter. And you'll know how many milliliters of solution that you added. And we know that the molecular weight of catalase is 240 kilodaltons or 240,000 grams per mole. So you can figure out how many moles of catalase protein you have from the concentration and that conversion factor. So that's something to note.

And then, once we have our amount of catalase protein, we can determine the amount of iron in the protein. So this is-- and we'll be doing that using the ferrozine assay. This is the structure of ferrozine. And the ferrozine molecule by itself is colorless. And then, when you complex it with iron, it turns into a magenta color.

And there are three molecules of ferrozine for every one iron ion. And so it forms in an octahedral sort of geometry. So, if there are six binding sites on iron, then you'll have one ferrozine here, one there, and one there. And it bonds to these two nitrogen centers here. And again, like the Coomassie dye, there are a lot of pi electrons and aromatic systems, which help to give it its color.

So the way that we're going to do this is we will be using again UV/Vis spectroscopy because we have something that goes from colorless to colored when it interacts with our molecule of interest or our ion of interest. So the first step in this procedure is going to be adding methanesulfonic acid. And then you will heat it at 104 degrees for 40 minutes.

And it's very important that you keep the temperature range around 104 degrees. And you don't want to go too high because we'll be heating them in the microcentrifuge tubes. And, if you heat it too hot, then your centrifuge tube can boil over, or it can build up pressure, and the cap will pop off. And you'll lose all of your solution. So you need to be careful with that.

And the purpose of this step is to release the iron from the heme group. So, when you add a bunch of acid, the iron gets released. And then we can quantify it because it won't be bound to our heme.

Then we will add sodium hydroxide to neutralize the acid. And then you're going to get a ferrozine complex mixture that will be prepared by your TAs. And you will add it to all of your samples. And the mixture is ascorbic acid, which reduces the iron 3 to iron 2+ because that is what will complex with our ferrozine molecule. Ammonium acetate helps to buffer the pH.

Neocuproine binds to any copper ions that are in the solution. So the ferrozine ligand is not necessarily specific to iron. So, if there are any copper contaminants, then it will also bind to copper, but the neocuproine is more specific for copper. So it will bind to the copper ions and make sure that it does not contaminate your assay. And then, of course, the ferrozine, that binds to the iron to make the colored complex that we are going to measure.

And then the-- so the measurement of the ferrozine is going to be pretty much the same as the BSA and the phosphate. Similar to UV/Vis, you'll make a bunch of standards of iron sulfate. And then you will have your catalase samples that have iron in them.

And then you will make your standard curve. The ferrozine is linear. So you'll be able to make your standard curve, just graph it, and use the line. And then you will be able to use the equation of your line to determine your concentration of your unknown samples from your catalase.

The data analysis for this lab is going to be using the protein assay results and the iron assay results. You will determine the number of iron centers per catalase. And so you have the micrograms per milliliter of your catalase protein. So you can convert that using your molecular weight to moles of catalase.

And then you will also get your iron in micrograms per milliliter. And, using the molecular weight of iron, you can convert that to moles and then do a mole-to-mole ratio of iron to catalase. And hopefully your answer is somewhere around four if all goes well.

So, in your discussion, you can talk about sources of error, how close you were to the theoretical value of four, any discrepancies between your calculated ratio, the expected ratio. And then you'll do an error analysis of the protein and iron concentrations. So you will run five unknown samples of catalase and five samples with the ferrozine assay of iron.

So you'll have five measurements that should be the same. So those you can do your average, your standard deviation, confidence interval. And then, if you have any outliers, that's when you can use the Q test for the outliers.

And there was a couple of questions that came up during the Charles River lab report. When we were making the standard curves for the phosphate determination, people had some outliers in their standard curves. And they were trying to figure out how they could get rid of those or if they should.

And the Q test only works if you're measuring the same thing multiple times. So that does not work for getting outliers out of a standard linear curve. But if you have-- in your standard curve, if you have one sample that is supposed to be a higher concentration than the one before it and it has a lower absorbance or something wildly higher, then you can look at it and say I know for a fact that, if something has a higher concentration, it should have a higher absorbance. I can get rid of this point.

You can also just kind of by looking at it-- that's not as scientific, but you can make an argument for why you would remove a data point based on how it looks compared to the line of best fit. And you can also compare the R squared values of the line with the point and without the data point. And, if it gets closer to 1 when you remove it, then it is more linear.

You obviously don't want to be taking out data points left and right until you only have like your three best points that give you a super straight line. But, if there are any that are very obviously not on the line, then that is one way that you can remove them.

So yeah, are there any questions about catalase or any of the other labs? Anything? This is all I have for today. This was kind of a short one. No.

All right, then those of you who are going to lab, you can head up there. And don't forget to turn your reports in. And yeah, the clipboard is around.