**Enzyme Science and Technology**
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**Module - VIII**
**Enzyme assay system and Kinetics**
**Lecture - 38**
**Enzyme Kinetic**

Hello, everyone. This is Dr Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati and what we were discussing? We were discussing about the different properties of the enzyme in the course, enzyme science and technology. And in the current module, we were discussing about how you can be able to measure the enzyme activity.

So, if you recall, we have said that or we have in fact, discussed that enzymes play crucial role in two different pathways. Either it is the anabolic pathway or the catabolic pathway. In the catabolic pathway, the enzymes are catalyzing the breakdowns of this multimeric substrates, such as starch or glycogen. And, that is how they are generating the glucose and from the glucose, it is oxidizing or degrading the glucose to carbon dioxide and water.

And, in this process, the catabolic reactions are withdrawing the energy, which is bound into the bonds and you know making the energy from these reactions. Apart from that, we also have the anabolic reactions where the enzymes are also you know synthesizing the new biomolecules. So, and both of these catabolic reactions or the anabolic reactions are very crucial for running the metabolism of the particular organisms.

Now, what we have discussed so far is that how the enzyme, you can actually be able to design the different types of assays, different types of radiometric assays, colorimetric assays, fluorometric assays and so on, and you can actually be able to measure the enzyme activity. But the question comes that how you can be able to study the enzyme kinetics or how fast an enzyme is actually forming the product?

So, in the today's lecture, we are going to discuss about the Enzyme Kinetics and how you can be able to determine the different types of enzymatic parameters and how you can be able to compare even the two different types of enzymes.

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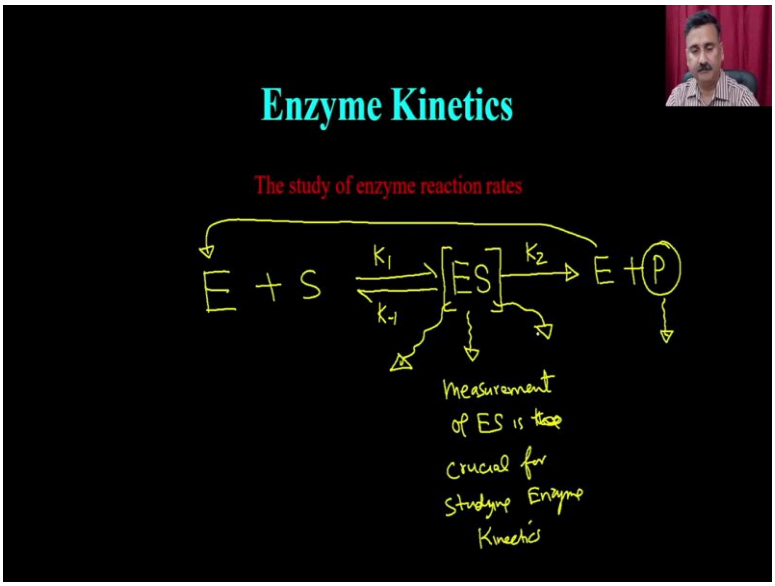


When we talk about the enzyme kinetics, the enzyme kinetics starts by the enzyme when it recognizes the substrate and it is forming the enzyme substrate complex. And, then that enzyme substrate complex, you know make the re-arrangement of the bonds and that is how the it forms the enzyme product complex. And then ultimately, since the product does not have the much affinity, it actually going to be released from the active site.

So, what we have is, we have the enzyme which is recognizing a substrate and, in this process, it is forming the enzyme substrate complex. As soon as the enzyme substrate complex is formed, it is actually going to be get fractionated into the two pathway. One, it is actually going to be fractionated that the enzyme substrate complex is going to be broken down into the enzyme and the substrate.

And, the second pathway is that the enzyme substrate complex is actually going to be form the enzyme plus product right. This product is actually was the readout when we were talking about the enzyme assays, right and while we were discussing the different types of methods to measure this enzyme assays, you can be able to say ok what is the enzymatic activity at that particular moment. But, when we talk about the enzyme kinetics, we have to see what are the different types of kinetic parameters, which are going to happen.

Now, once the enzyme is actually going to be released, if this enzyme is actually going to come back into the this state right and that is how it is actually going to participate

into the another round. And, that is how this cyclic events will continue and that is how you are going to have the large quantity of the product which is going to be accumulated.

Now, the major question is in the enzyme kinetics is that imagine that the enzyme is interacting with the substrate and it is having the rate constant of K1 with it is which is catalyzing and then it is forming the ES. Now, when you have the breakdown of these ES, you can actually have the K minus 1, right. And, that is actually going to denote the rate constant for the ES breakdown into the E plus S. Whereas this K, this can be K 2 which is actually going to be the breakdown of or the convergent of ES into the enzyme plus product.

Now, the first and the most important question is that if you want to calculate or if you want to study the enzyme kinetics, you have to actually measure the concentration of the enzyme substrate complex, right. And, enzyme substrate complex measuring the enzyme substrate complex is the bottleneck of the enzyme is studying the enzyme kinetics, right. So, measurement of ES is the is crucial actually, right it is crucial for studying the enzyme kinetics.

Now, the question comes how you can be able to measure the enzyme substrate transition state right or enzyme substrate complex for measures because the timeline of the half-life of this particular complex is very small, right. And, that is how you cannot be able to do any kind of you know the analytical methods.

So, you cannot actually isolate this enzyme substrate complex or you cannot actually trap the enzyme-substrate complex, so that you can be able to measure, right. And, that is why if you cannot measure the enzyme-substrate complex, you have to make the different types of assumptions and different types of assumptions to measure the enzyme-substrate complex.

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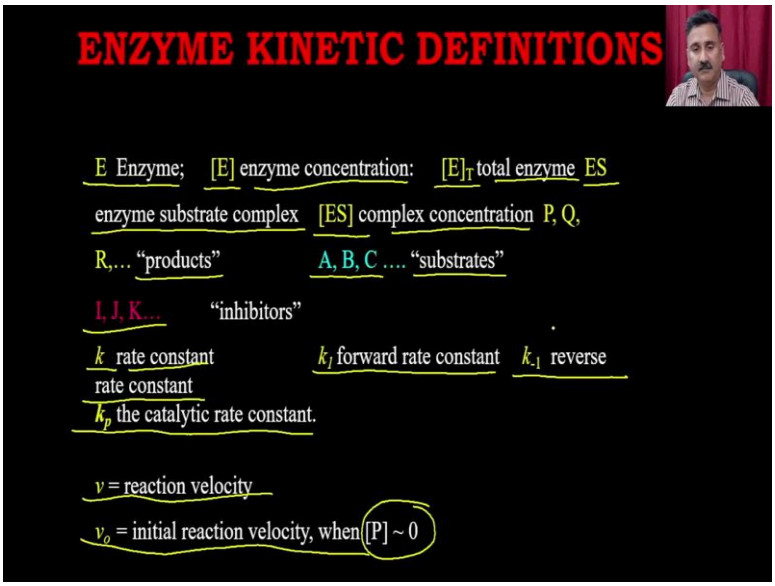


But, before getting to detail of these issues, let us understand that in this particular discussion what are different types of symbols we are actually going to use so that it is easier for you to follow the content. What we are going to say is when we are going to say E, it is actually going to enzyme; when we say E bracket, it is actually going to denote the enzyme concentration.

When we say E T that is the total enzyme, ok, total enzyme; when we say ES, it is actually going to say the enzyme-substrate complex. When we say ES in the bracket, it is going to called as enzyme-substrate complex concentrations. P, Q, R are the letters which are actually always being used. For denoting the products, whereas, the A, B, C can be used for the substrates.

Then you can also use the I, J, K that is for the inhibitors and then you can also use the k, which is for the rate constant. k 1 is actually going to be the forward reactions. k minus 1 is actually going to be the reverse reactions. k p is actually the catalytic rate constant or the k 2. Then v is the reaction velocity and v 0 is the initial velocity when the product formation is 0.

So, after getting into the detail of these enzyme-kinetics definitions, we will come back to the same problem that you are actually going to or you are supposed to measure the enzyme-substrate complex. And, there is no way that you can be able to measure the enzyme-substrate complex concentrations because it is difficult to isolate. You cannot

have any kind of the techniques so that you can be able to isolate the enzyme-substrate complex.

And, on the other hand, the enzyme-substrate is a very, very transient, right. So, it actually going to be formed and then it is actually going to be broken down. So, that is why it is difficult to measure the accurate amount of enzyme-substrate complex. So, if we cannot measure the enzyme-substrate complex, you have to make the different types of assumptions in case you want to study the enzyme-kinetics.

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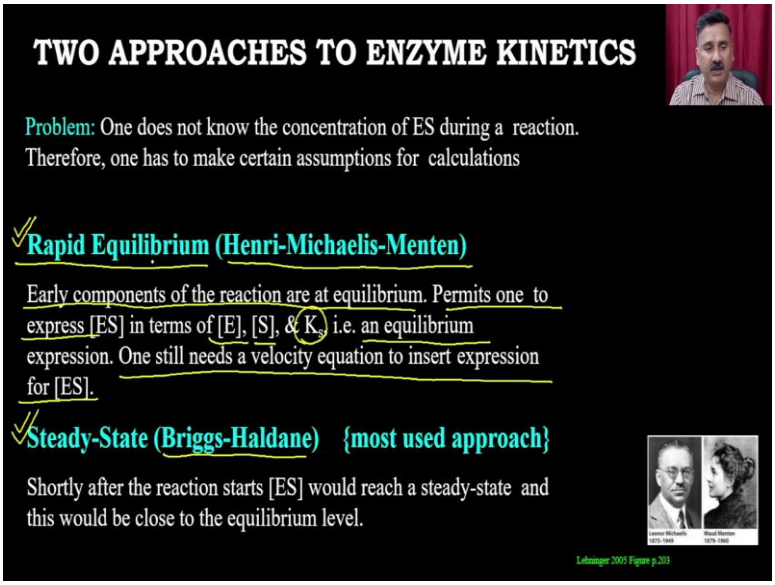


So, we have two different types of assumptions. One is called as the rapid equilibrium which is the assumption being made by the Henri-Michaelis-Menten and the second is the steady state assumptions, which is being done by the Briggs-Haldane. And, this is the latest approach. This is the older approach.

So, what is the rapid equilibrium? In the rapid equilibrium, the early components of the reactions are at equilibrium permits one to express the enzyme-substrate complex in terms of the enzyme concentration, substrate concentration and the K S which is an equilibrium expressions. So, one still need a velocity equation to insert the expression for the enzyme-substrate complex concentrations. So, let us first discuss about the rapid equilibrium, right.

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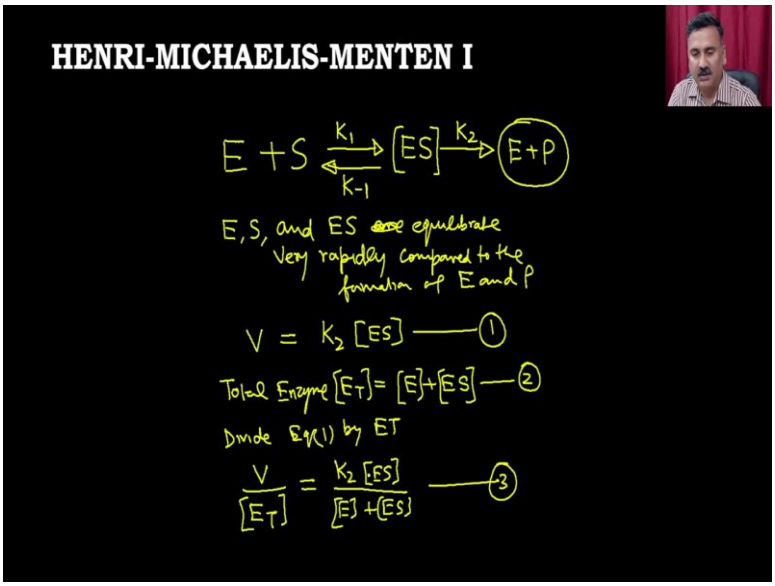


In a rapid equilibrium, what you have is you have this, right. So, enzyme is interacting with the substrate, right and then it is actually going to have the forward reaction which is going to be responsible for the formation of the enzyme-substrate complex and the rate constant for this is actually going to be K 1.

Then this is actually going to be broken down into the enzyme plus product, right and this is going to be called as K 2. Now, enzyme-substrate is actually going to be broken down into the enzyme and substrate, right at the equilibrium right. So, it is actually going to have a rate constant of K minus 1.

Now, E, S and E S, so, as soon as you start the reactions in the initial time points, the ES and ES are equilibrate right. So, it is actually going to have the equilibrate, very rapidly, right compared to. So, it is going to be equilibrate very rapidly, right. So, at the initial time points, it is E, S and ES are actually going to be under the equilibrium because there will be no product which is going to be formed rapidly compare to the formation of compare to the formation of E and P, right.

So, until the E and P is not being formed, the E, S and ES are actually going to be under the equilibrium. That is why the velocity of this reaction is going to be called or velocity of this reaction like the formation of the ES concentration right. So, ES formation of ES is the velocity to form the ES is actually going to be that you can actually have the K 2 and the concentration of E, S right. So, this is called as equation 1.

Now, you know that the total enzyme. So, the total enzyme E T is. So, you have the enzyme here, you have the enzyme here. So, you can actually be able to add this, right. So, E plus ES, right. So, that is the total enzyme, but it is being present at this particular moment when the velocity is K 2 ES. So, if you add this enzyme question number 2.

Now, if you divide the equation 1 to by the ET, right. So, what will happen is that it is actually going to give you the V by E T and this is the concentration ok. So, we are talking about the total concentrations ok, and which is equivalent to the concentration of the enzyme and concentration of the enzyme-substrate complex.

So, remember that we our main objective is to calculate the concentration of the ES, right, and by doing this we are actually doing this, right. So, then it is going to be K 2 ES divided by, and instead of writing the E T, I am writing this E plus ES, right. So, it is going to be right E plus ES right, and this is the equation number 3. Now, let us move to the next step, right.

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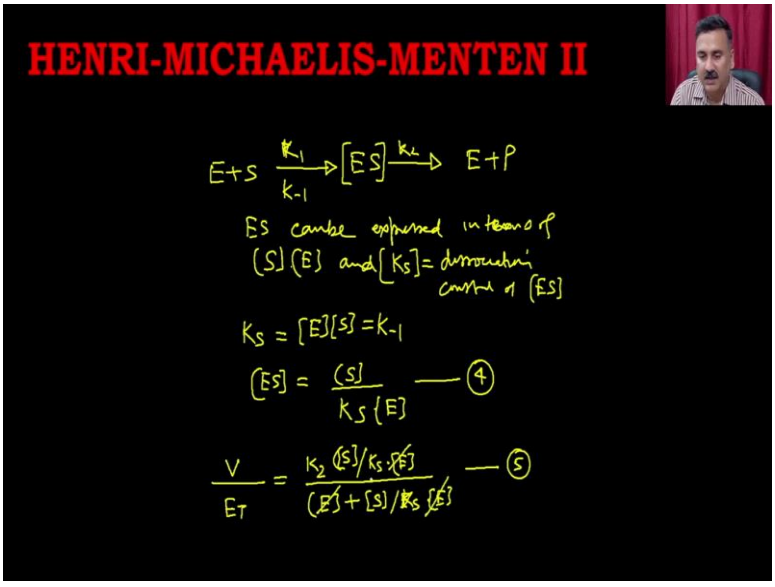


So, next step is, we will write here again. So, so E plus S is K 1 right, and it is going to form the E S, and K minus 1, and K is going to be K 2, E plus P, right. So, since the equilibrium is very rapid right, ES can be expressed. So, ES can be expressed in terms of, in terms of S, right E and K S. K S is the dissociation constant, K S is the dissociation constant of ES, right.

So, you can write K S, which is equivalent to ES, ok. Since the ES, the concentration of ES is equivalent to the S divided by K S into E, right, ok. So, this is the going to be the equation number 4, right, and if you substitute the value of E S into equation 3. So, remember the equation 3 here, right. So, this is the equation 3, right.

So, if we take this equation and put the values of the ES, this value in this equation, right, then what you are going to get is, you are going to get the V by E T equal to K 2 bracket [FL] S by K S into the total enzyme configuration of E, right, divided by E plus S by K, sorry, K S into E, right. And, in this the E is actually going to be cancelled out, right. So, ultimately so, this is equation number 5, ok, and in this you will see that the E is can be cancelled out from this, right, and ultimately this is going to be equation number 6.

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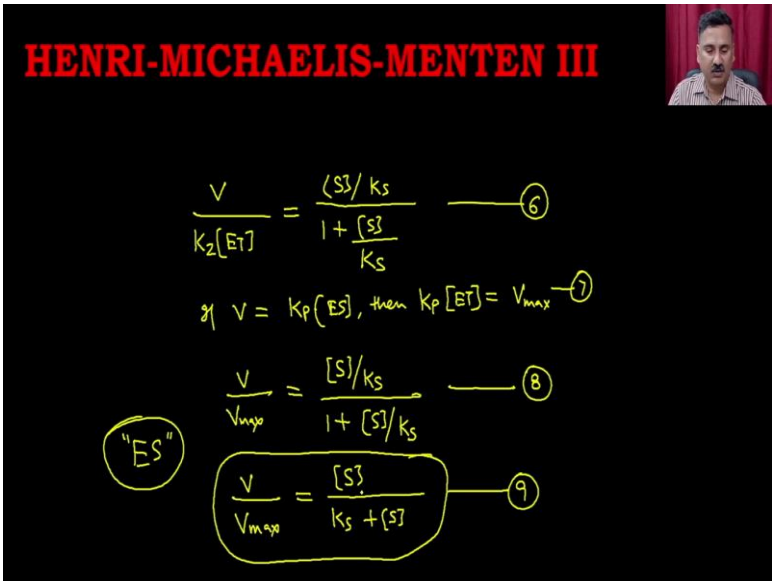


Now, once you have this, you can actually be able to say this, right, V is equal to V by K 2 E T, ok, which is equivalent to. So, if you cross multiply both the side by K 2 and cancelled out E on the right side, it is actually going to give you the V by K 2 by E T, that is equivalent to S divided by K S divided by 1 plus S by K S, ok. And, if V is equivalent to K P into ES, right, then K P ET is going to be the V max, ok, because the enzyme is going to be get saturated at this particular level.

So, this is the equation number 6, this is the equation number 7. So, now, if you take the equation number 6 and 7 and you will put all these values, then it becomes V by V max equivalent to S by K S divided by 1 plus S by K S, ok, and if you simplify. So, this is

going to be the equation number 8 and if you simplify this, it is actually going to say that V by V max is equal to S divided by K S plus S and this is going to be the final equation, what you can actually be able to use and you can be able to calculate the enzyme kinetics.

Now, if this is this equation has a lot of significance because this equation can give you the many types of answers, right. Remember that in this particular equation, you can be able to still be able to calculate the enzyme velocity, you can actually do lot of calculations, but there is no term of ES.

So, so we are actually get rid of this ES term simply by expressing the ES in terms of the S, ES and S, E and other kinds of parameters. Now, see how we can be able to use this equation to understand the different aspects of the enzyme kinetics.

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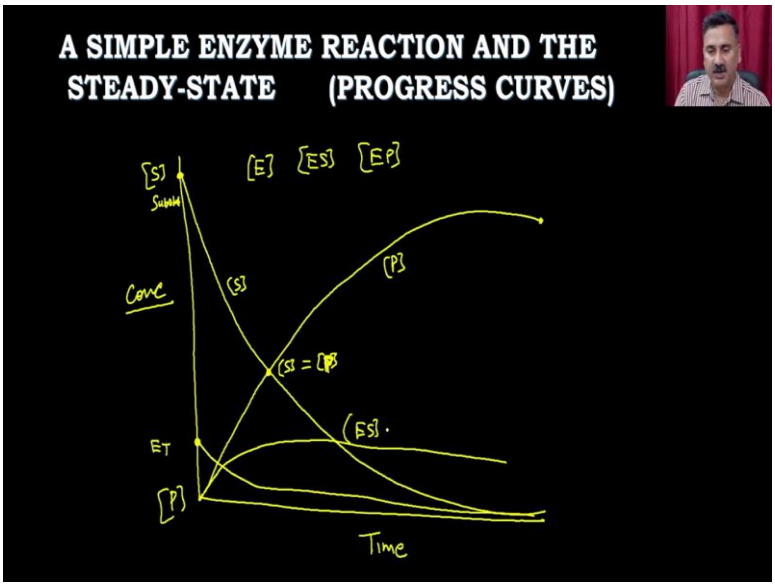


So, if you plot, take the plot, you take the time on one angle and the concentration of the different species, right. So, if you take the concentration on this side, what will happen is that your enzyme? So, first talk about the concentration of the substrate ok. Now, substrate is going to be very high in the beginning, right and then ultimately it is actually going to come down, right, because the substrate is going to be utilized and that is how the substrate.

So, substrate concentration is going to be very high, right, whereas, it is actually going to be come down, right and ultimately it is going to be around to 0. Similarly, for the product, like if you see the product. So, at this particular time the product is going to be 0, but as you are actually going to increase, it is actually going to increase, right. So, this is here, the product is going to be the maximum and where the place you will see, this is the this is the curve of the substrate, this is the curve of the product and you see the intersection, right.

At this intersection, the concentration of the product is actually going to be equivalent to the concentration of the so, concentration of the substrate is equivalent to the concentration of the product. Let us also see how the enzyme is actually going to be get saturated, ok. So, initially you are going to start with this amount of enzyme which is actually going to be the total enzyme and it is going to be the enzyme only.

Now, as the reaction will proceed, the enzyme is actually going to be get fractionated into the 2 3, this species, it is actually going to be E, which is the plain enzyme, it is going to be ES and it is also going to be the E P, right, and you will see that the initially the enzyme is going to be the total enzyme, right, which is like this and then it is actually going to come down, right.

And, that in reduction in the enzyme is actually going to be used up in terms of the formation of the enzyme substrate complexes, right. So, this is the curve for the enzyme substrate complex, right. But, at this point the enzyme substrate formation and the conversion of the enzyme substrate into the enzyme is also going to be the same, ok. So, this is all about the simple reaction and how the modulation or the dynamics or the different types of species which are involved into the enzyme reactions are going to be change.

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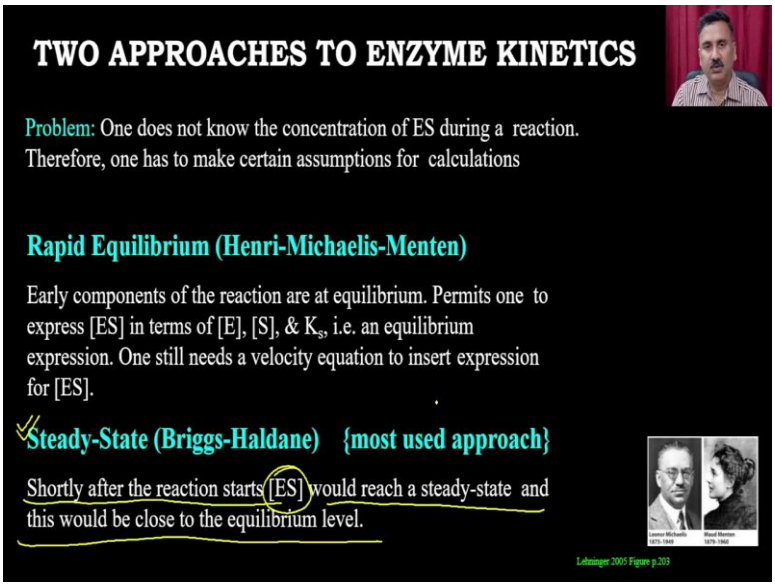


Now, let us go to the next approach, the next approach is called as the steady state Briggs-Haldane approach. So, shortly after the reaction starts when the ES is actually going to start forming, would reach a steady-state and this would close to the equilibrium level, ok. So, the steady-state assumptions are more accurate, they are actually going to give you the better picture of how the enzyme is actually going to catalyze the reactions, ok.

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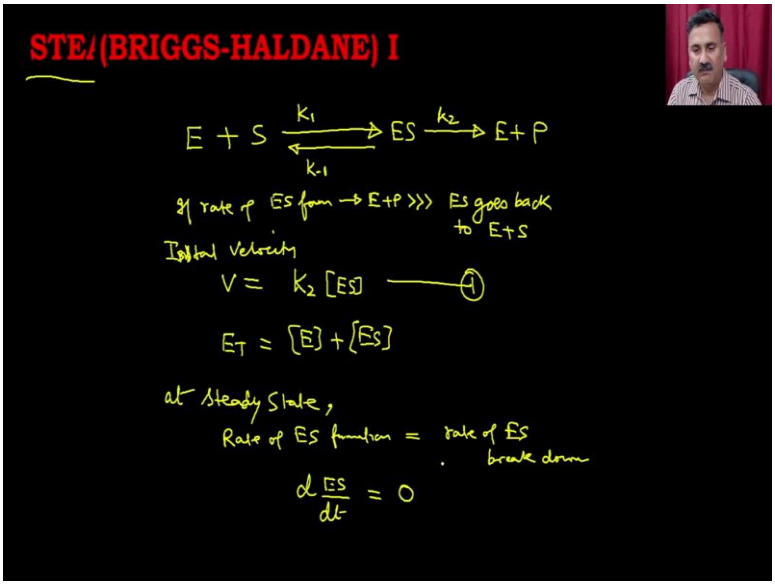

So, what happen is that we will write again the same reactions, right. So, we will write the enzyme plus substrate, then it is actually going to form the enzyme substrate complex and then it is actually going to form the enzyme plus product, right. And, then you will also have the breakdown of the enzyme substrate complex and the rate constant for this is going to be K 1, rate constant for this one is going to be K minus 1 and this is going to be K 2, right.

And if, now you can imagine that if the rate of ES formation, it is actually going to give you the E plus P, right, and if the E plus P is very big, then the ES can be calculated even from the ES goes back to E plus S, ok. So, if the ES formation is getting converted into the E plus P, then the ES is getting reverse, right, and it is actually going to form the E plus S.

Now, you see that the, what will be the instants velocity, ok. So, instant velocity is V, V is equal to K 2 and the concentration of ES, ok. So, this is going to be the instant velocity. Now, this is going to be the equation number 1, right, and you know that the E T is going to be E plus ES, right. So, and you know that at the steady-state concentration, the concentration of ES is going to be the constant, right. So, at steady-state means the ES, how much ES is forming, the ES is getting breakdown, right.

So, that this means the ES is actually going to be under the equilibrium, which means the rate of ES formation is equivalent to the rate of ES breakdown, right, and this means d ES by dt is going to be 0, which means it is not going to change, right. So, there will be no change in the rate of formation, right, and the rate of ES formation is going to be the equivalent to the rate of ES breakdown. Now, taking this into the account, you can be able to calculate or you can be able to derive the kinetic equations.

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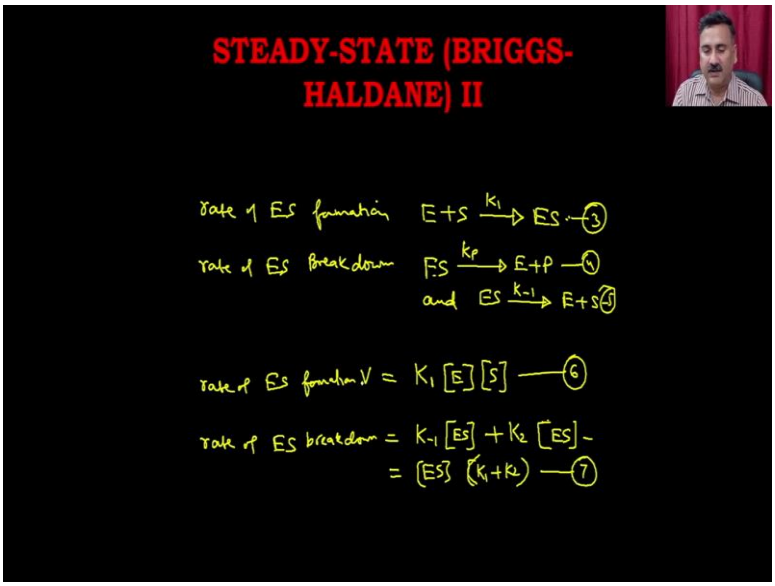


So, what will be the rate of ES formation, right? So, you know that E plus S giving rise to ES, right, that is the rate of formation, right. So, we can write. So, this is going to be the equation number. So, this is K 1, right. So, this is going to be called as equation number 3 and the rate of ES breakdown, that is going to be ES giving rise to E plus P and that is going to be calculate by the K P, right.

And, ES giving rise to E plus S and that is going to be calculated or catalyzed by the K minus 1, right. So, if you write the rate of ES formation, so, that you will write equal to K 1 and the concentration of E to the concentration of S, ok. So, this is the rate of formation, right, because you can actually have the K 1. So, this is going to be the velocity.

Then rate of ES breakdown is going to be this, and this, right. So, you can write K minus 1 ES plus K 2 ES and if you want, you can take the ES common, right. So, it becomes K 1 plus K 2. So, that is the K 1, K 2 and this is going to be. So, this is going to be the. So, this is the equation number 4, this is the equation number 5, this is going to be the equation number 6 and this is going to be the equation number 7, ok. So, we know that rate of ES formation and rate of ES breakdown, now you can just make it equal to each other, right.

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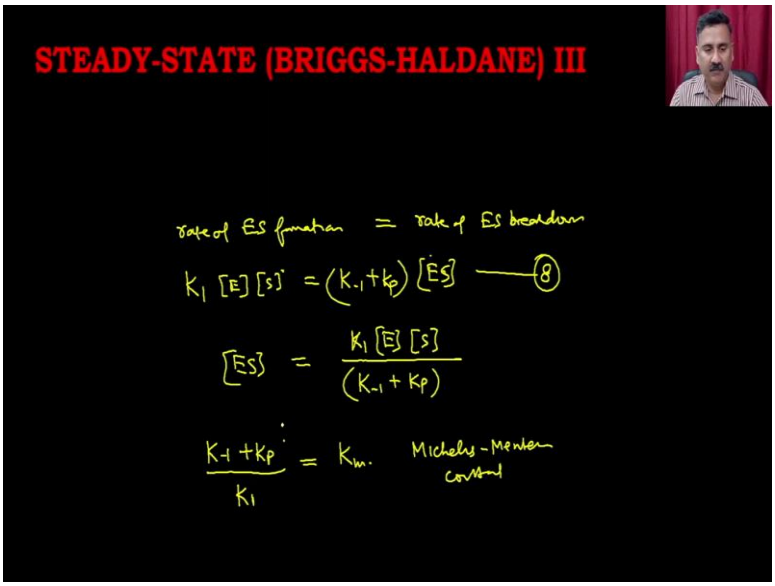


So, rate of ES formation. So, add a steady state, rate of ES formation is going to be equivalent to the rate of ES breakdown, right. So, just right here, right. So, K 1 E S is equal to K minus 1 plus K P and rate of ES, concentration of ES, right. Now, this is going to be the equation number 8. Now, if you solve the equation number 8 for ES, right.

Then the ES is going to be the, if you solve this equation, the ES is going to be K 1, E S divided by K minus 1 plus K P, right. Now, K 1 plus K P upon K 1, so, this one, right so, K minus 1 plus K P divided by K 1 is going to be called as K m or it is called as Michaelis – Menten constant, ok. This means you can actually be able to use this instead of this, right, and you can be able to calculate, right.

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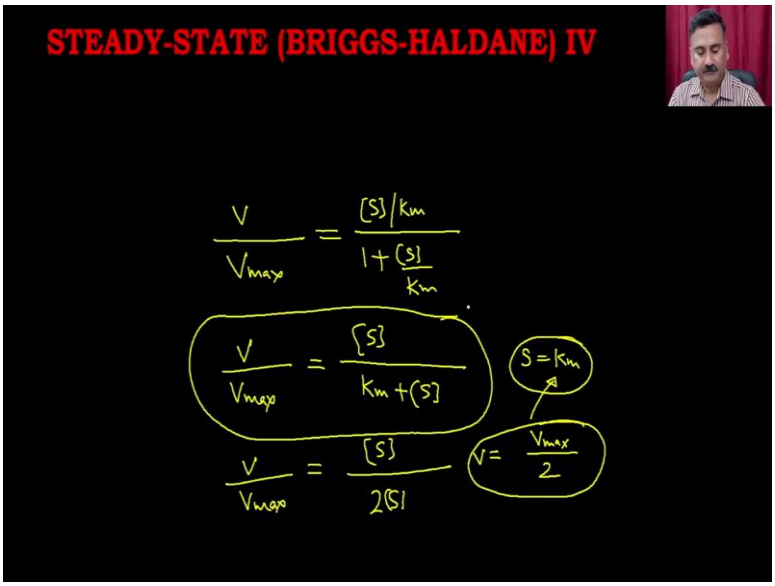


So, if you put all this you will get this, ok, you will get the V by V max is equal to S divided by K m, divided by 1 plus S by K m, ok, or you can write like this, V by V max is equal to S divided by K m plus S, ok, then you can bring this down and you can, if you rearrange, it is actually going to give you this, ok, and this is a very, very important equation, ok.

Now, you can imagine a situation when the S is equivalent to the level of K m. So, if you increase the concentration of the substrate which is equivalent to the value of K m, then what will happen? You see the V by V max is equal to S and instead of K m you can write S. So, it becomes 2 S, right, this means V is going to be. So, if you divide this, ok, what will happen is that the V is actually going to be V max by 2.

And, that is a very, very, very important clue to calculate the value of K, which means if you calculate the velocity of the enzymes, wherever you will find the velocity is the velocity of V max, it is actually going to give you the value of K m and that is how it is very, very important. So, this equation is called as Michaelis – Menten equation.

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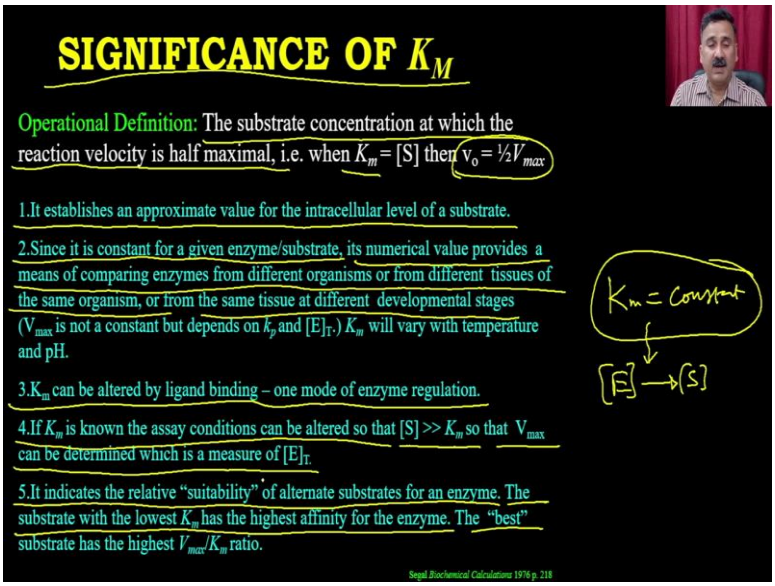


Now, what will be the significance of the K M into the enzyme kinetics? The substrate concentration at which, so, this is the definition of the K M. The substrate concentration at which the reaction velocity is half of the maximal, that is the K m is the substrate when the initial velocity is half of the velocity, maximum V max, it is going to be called as K m.

It is establishes the approximate value for the intracellular level of substrate, this means if you want to attain the velocity of the half of the maximum velocity, you should have the substrate concentration which is equivalent to the K m values. Since it is a constant for a given enzyme substrate, it is numerical value provide a mean of comparing the enzyme from the different organism or the different tissue of the same organism or from the same tissue at a different developmental stages.

So, K m is a very, very important parameter because K m is a constant, right and it is constant for the particular enzyme substrate combination, right. So, that is why K m is a very, very good parameter to compare the same enzyme from the different sources, same enzyme from the different organisms and even the same enzyme under the different developmental stages.

Like for example, if you talk about the lactate dehydrogenase, right. So, that LDH is an enzyme which is present in the liver, it is present in blood, it is present in the heart and other places. So, you, but the activity of the LDH is different in their different activity

right. So, you can be able to calculate the K m of the LDH and that is actually going to give you a significance in terms of who is going to be more active and who is going to be less active because if the K m is lower, it is actually going to be a better enzyme.

K m can be altered by the ligand binding, which is one way of enzyme regulation, right. So, it is not like the K m is constant, it is can be altered when you alter or when you make the modifications into the enzyme. If the K m is known, the assay condition can be altered so that the substrate concentration is going to be above to the K m so that the V max can be determined, which is a measurement of the total enzyme.

It indicates the relative suitability of the alternate substrates for an enzyme. The substrate with the lowest K m has the high affinity for the enzyme. The best substrate has the highest V max by the K m ratio.

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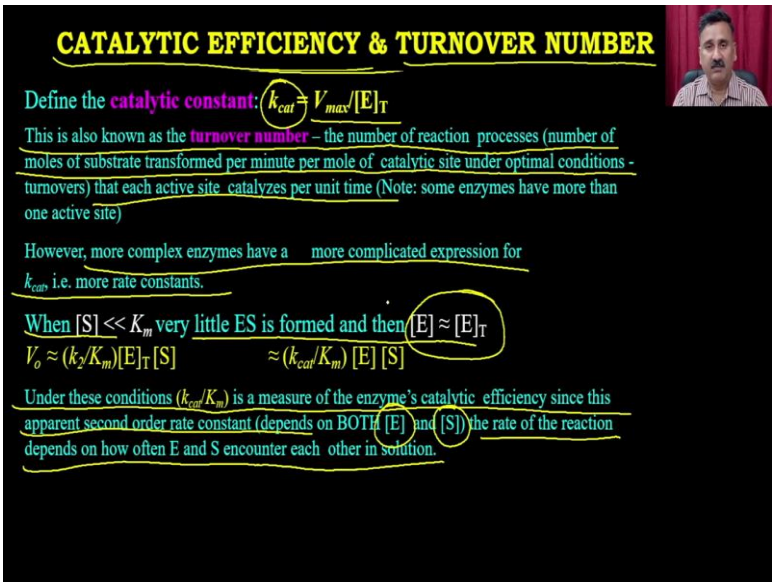


Now, apart from the K m, you also have the two more parameters which actually can be used to calculate or to compare the enzyme. One is called as the catalytic deficiency; another one is called as the turnover number. So, what is the catalytic constant? Catalytic constant the k cat is called as V max by the total enzyme.

So, this is also known as the turnover number – the number of reaction processes, the number of moles of substrate transformed per minute per mole of catalytic activity, catalytic site under the optimal condition that each active site catalyzes per unit. It is

important that you should know that the enzyme may have the more than active sites and in that case the calculation has to be done accordingly.

However, more complex enzyme have a more complicated expression for the k cat. When the substrate is very very low compared to the K m, that is the very little ES is formed, then the enzyme is equivalent to the total enzyme. And in that case, it is actually going to be give you the catalytic efficiency.

Under these conditions, the k cat by K m is a measure of the enzymes catalytic efficiency. Since this apparent second order rate constant, it depends both on the enzyme and as well as on the substrate, the rate of the reaction depends on the how often E and S encounter each other in the solution. So, both the catalytic efficiency and the turnover numbers are very, very important parameter which you can actually be able to derive from the enzyme kinetics. And, that can be used to compare the two different sources of the enzyme.

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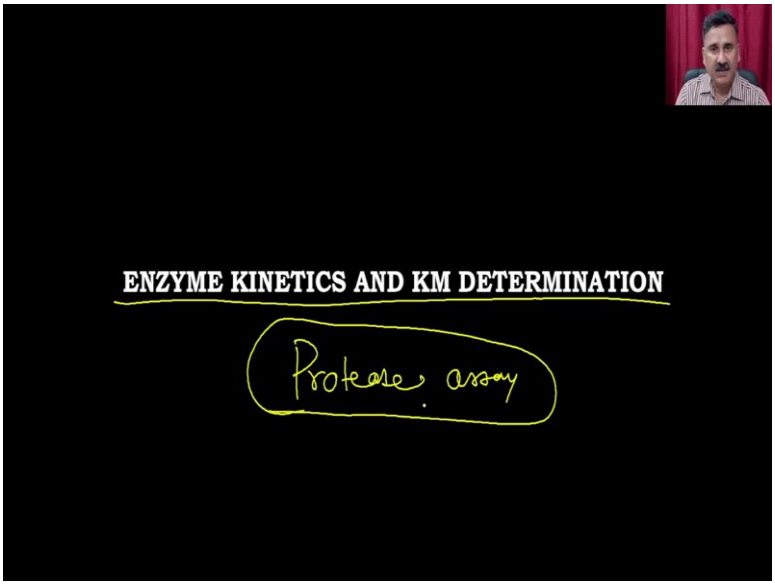


Now, let us talk about how you can be able to determine the K m ok. So, if you want to determine the K m of an enzyme for a particular substrate, you have to perform the enzyme assays. So, in this particular examples, what we have done is we have taken an example of a protease assay where we are actually going to you know provide the substrate to the protease and we will perform the protease assay and at the end, we are actually going to calculate the K m of the substrate for the enzyme.

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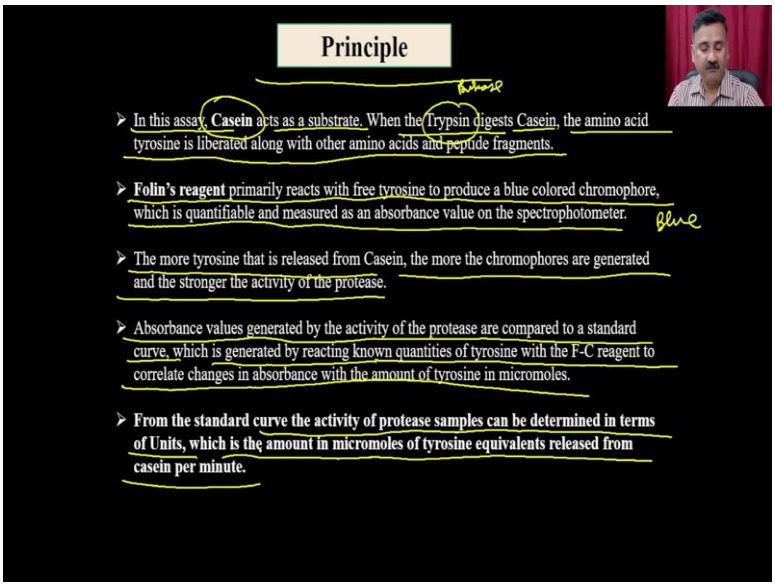


So, what is the principle of this particular assay? So, in this assay, the casein, which is a protein is act as a substrate. When the trypsin which is actually a protease. So, this is actually an enzyme, this is the substrate, digest the casein, the amino acid tyrosine is liberated along with the other amino acid and peptide fragment. And, then you can, since the tyrosine is being liberated, you can actually be able to use the folin's reagent to detect the free tyrosine, which has been which and it is actually going to give you the blue color reactions.

And, you can actually be able to measure that using the colorimetric assays and it is going to give you the blue color, right. Now, you can imagine that if the more tyrosine is released from the casein, the more chromophore are generated and the stronger the activity of the protease.

So, what you can do is you can calculate the absorbance values generated by the activity of the protease and compared it to the standard curve which is generated by reacting the known amount of tyrosine with the folins ciocalteu reagents to correlate the changes in the absorbance with the amount of tyrosine in the micromoles. From the standard curve, the activity of protease sample can be determined in terms of the units, which is the amount of micromoles of tyrosine equivalent released from the casein per minute.

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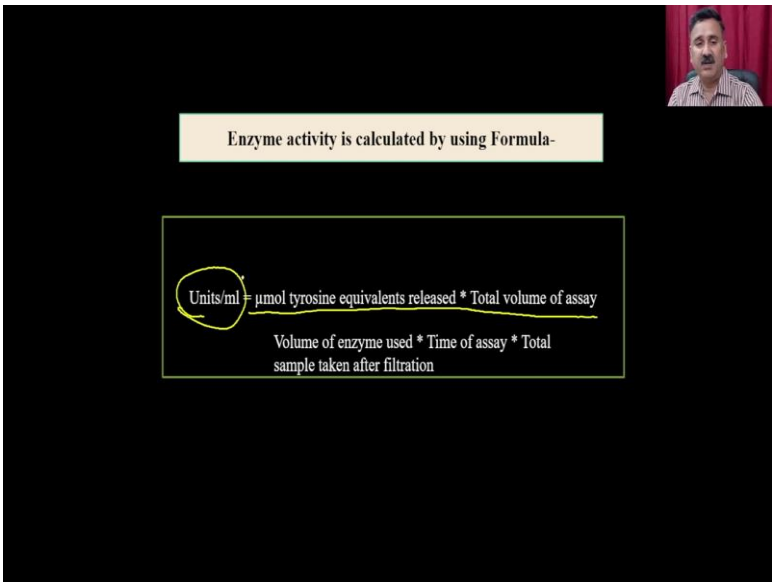


So, the activity what you are going to determine it is actually going to be calculated in the units per ml that is the micromole of tyrosine, which is going to be released. And you can use this formula to calculate the units.

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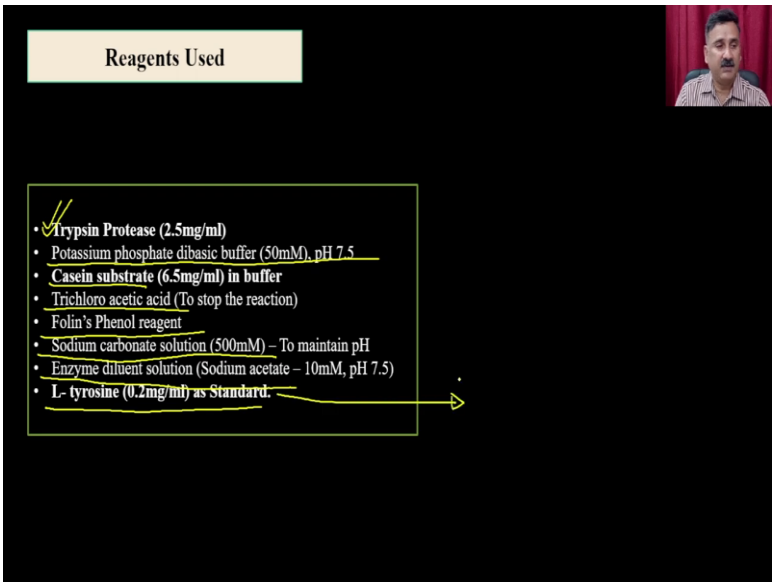


If you want to do this experiment, what are the different types of reagents are required? So, you require the trypsin which is actually the protease, you require the buffers. So, you require the phosphate buffers, you require the casein, you require the TCA, you require the folins ciocalteu reagents, you require the sodium bicarbonate, enzyme

diluents and then you also require the L tyrosine which is going to be the standard (Refer Time: 42:11) And, how you are going to perform this?

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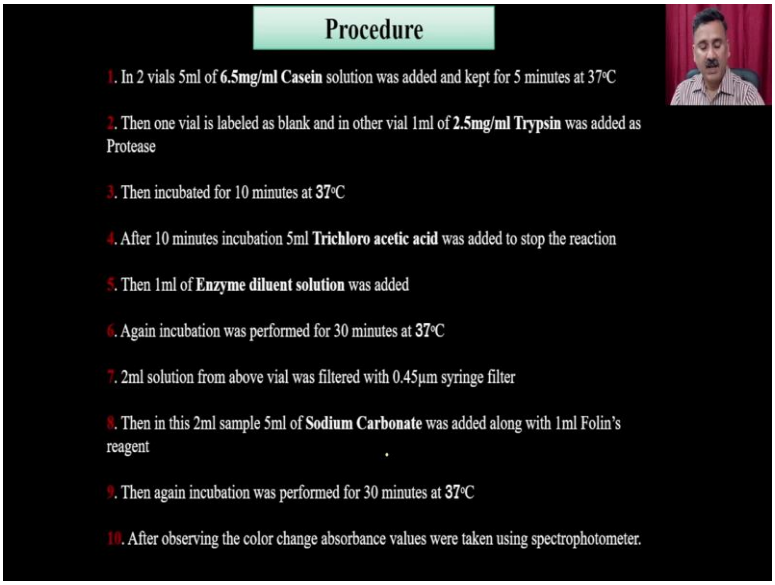


So, you are going to follow this particular procedure. So, where you are going to incubate the casein with the enzyme and other kinds of reactions and you are going to follow this particular reactions and then ultimately you are going to have the you know the tyrosine standard curve.

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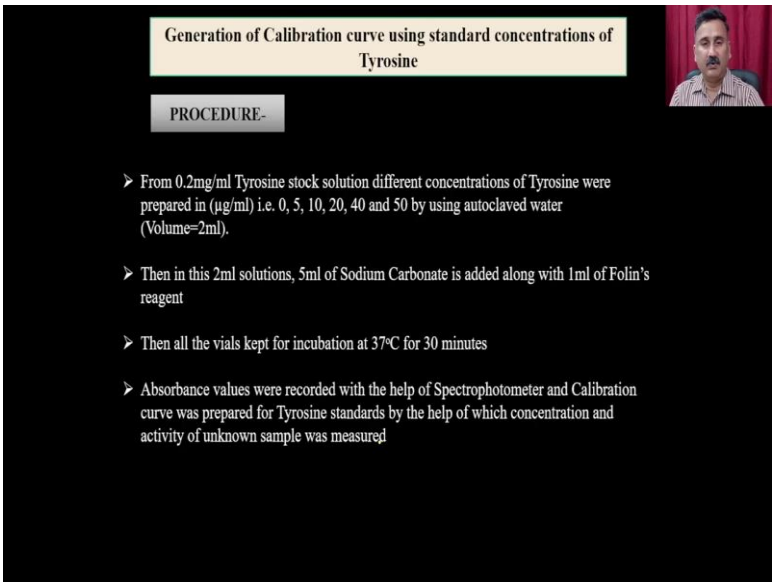

So, you are going to run the tyrosine standard curve and that is how you can be able to calculate the amount of tyrosine which is going to be liberated. In every reactions what you are going to perform and ultimately you can be able to plot that in terms of calculating the K m values.

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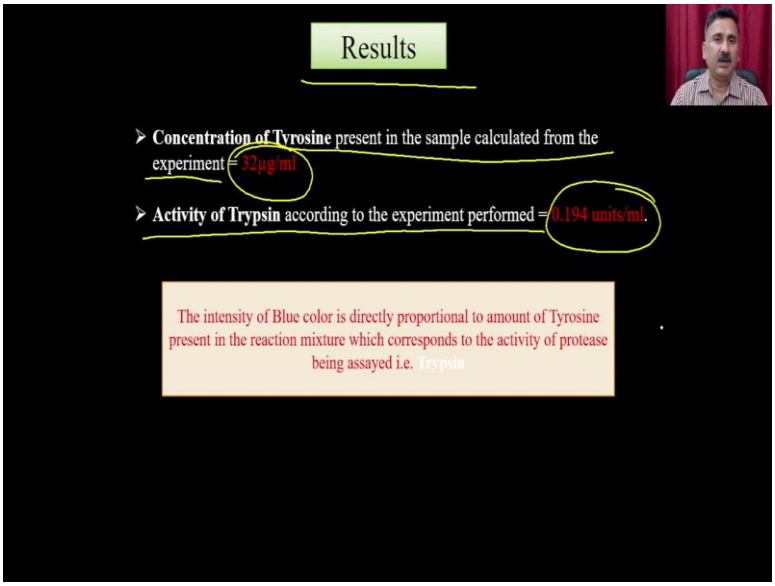


Now, the result what you are going to get, so, the concentration of tyrosine present in the sample calculated from the experiment is going to be this and you can actually be able to use this to calculate the activity of trypsin according to this. It is actually going to be this, right.

Now, how you are going to calculate the K m values? So, K m actually determination of K m by using this kind of data is having you can have the two options. One you can actually be able to do the Michaelis – Menten curve and you can be able to calculate the K m values or you can actually be able to have the Lineweaver plot.

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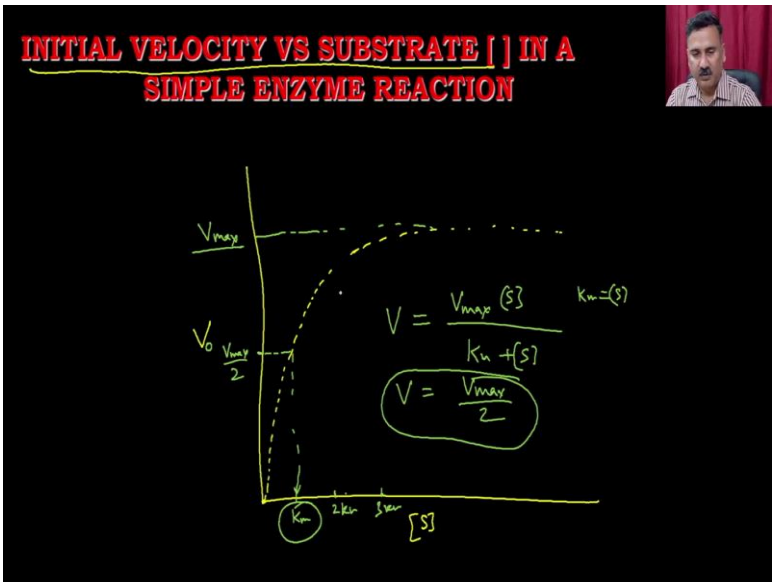


So, when you are actually going to plot the initial velocity versus the supernatant it is actually going to give you the Michaelis-Menten curve. So, what you are going to do is you are going to do the substrate concentration on the x-axis and you are going to plot the velocity on the y-axis.

And, when you are going to do this what you are going to see is you are going to see that the initially the velocity is going to be logarithm linear and then ultimately it is actually going to form the platform, right. Because with time with increasing substrate it is not going to increase because now at this particular stage the enzymes are saturated, right.

And this is actually going to give you the. So, this is actually going to give you the V max ok. So, because this is the maximum what you got right. So, this is the V max, right. Now, and this is the initial velocity, right, what you have plotted against the substrate concentrations, right. And, remember that this is the Michaelis-Menten curve equation right V max concentration of substrate divided by K m plus S, right and remember that is.

So, V max we got the V max, right and what you can do is you can just go by the half of V max, right. So, this is suppose this is the half of V max right. So, V max by 2 and you if you go like this it is actually going to give you a concentration of the substrate and that is going to be the K m, right. If you remember right, we have put that if you if the K m is equal to the substrate concentration, then V is actually going to be half of V max, right.

Same way you can have the 2 K m, you can have 2 K m, you can have 3 K m like that ok.

And that is how you are actually reaching to a saturation point. So, this is one of the way in which you can be able to draw the Michaelis-Menten curve and you can be able to calculate the K m values. Now, the question is that the K m in this is actually going to be on the assumption that this is you know so, this kinetics is less reliable because of the so many issues ok. So, that is why people are now preferring that we you should also go with the Lineweaver plot.

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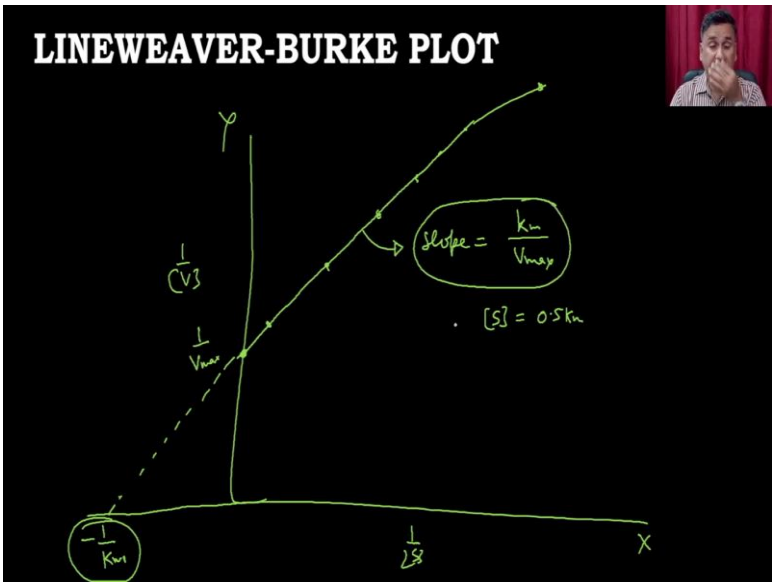


So, in a Lineweaver plot what you are doing is you are actually going to do the plotting of 1 by S versus 1 by V, right. And in that case what you are going to do is you are going what you are going to get is you are going to get the curve like this ok. Because remember that in this particular curve we are actually making the assumption of V max simply by going with the saturation curve, right. But, that may or may not be correct, right. So, in that case your calculation of the K m is not going to be accurate.

And to make the things more accurate people are going with the Lineweaver plot where you are not going to have any saturation. And this is the point where it is actually hitting the y-axis is actually the 1 by V max ok and this is the 1 by V max. And if you have the different time points right different concentrations right of the substrate you are actually going to have these values.

And, when you extrapolate this curve, it is actually going to hit the x-axis. So, x-axis this is the y-axis. And this is the value what you are going to get 1 by minus 1 by K m ok and, if you take this value and you can be able to calculate the K m values. So, if the substrate is at 0.5 K m right, it is actually going to give you the maximum saturation maximum point.

Whereas for the slope you can be able to calculate the K m by V max ok and that is also very very important parameter to compare the two different types of enzymes. So, to explain you much in detail how you can be able to do the protease assay how you can be able to use that data to calculate the Michaelis-Menten constant we have prepared a small demo clip. And, in this clip the students have explained you that how you can be able to calculate the K m from the velocity data.

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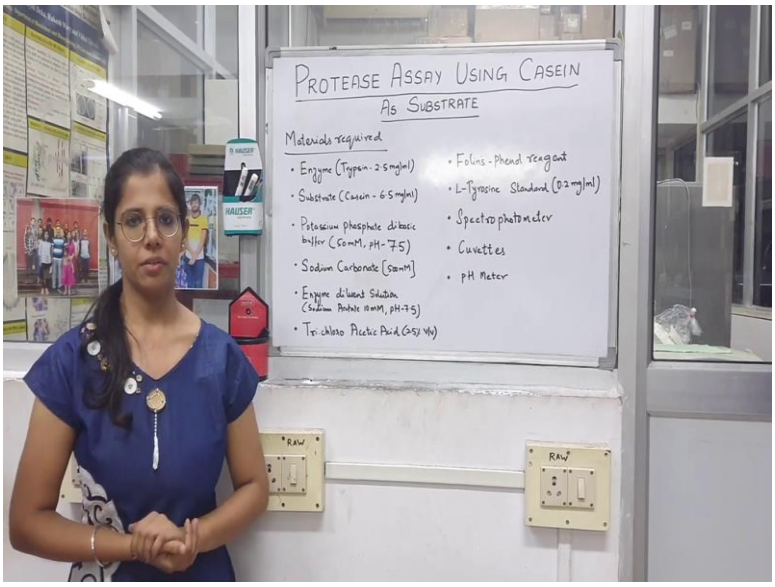


Hello, everyone. This is Eena Dodwani from Malaria Research group IIT, Guwahati. Today in this video we will be performing protease assay using casein as substrate. Many other proteins can be used as substrate that is BSA, gelatin etcetera. But we will be using casein because casein is easily available and stable under storage conditions.

We are using casein as substrate and trypsin which is a serene protease whose enzyme activity is to be determined. The basic principle for this experiment is S proteases called peptide bonds. So, it is necessary to measure and compare the activity of different

proteases. Here in this experiment trypsin which is serene proteases digest, casein and releases amino acid tyrosine along with other amino acids and many peptide fragments.

Folins, phenol reagents detects free tyrosine in the reaction and give produces blue color chromophore. The more the number of tyrosine produces in the reaction, more will be the chromophores generated and more intense will be the blue color. The intensity of blue color is directly proportional to the activity of proteases.

Absorbance value obtained from the activity of proteases are compared with standard curve which is obtained with tyrosine known quantity of tyrosine reacted with folins reagent. The change in absorbance is mainly correlated with amount of tyrosine in micro moles. We can determine we can determine the activity of proteases with the help of plotted standard curve.

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For the preparation of standard curve, we will be using different reagents such as potassium phosphate buffer having 50 millimolar concentration and pH 6.5; sodium carbonate buffer having 500 millimolar concentration; enzyme diluent solution that is sodium acetate buffer having concentration 10 millimolar and pH 7.5; TCA that is trichloroacetic acid 25 percent.

Folins phenol reagent and here trypsin having concentration 2.5 mg per ml which will be which we will be using as protease; L tyrosine which we will be using as standard having

concentration 0.2 mg per ml and casein having concentration 6.5 mg per ml which is our substrate.

For this experiment we have to prepare a standard curve. For this we need different concentrations of tyrosine that is 5 micrograms per ml 10, 20, 40 and 50 micrograms per ml. For the sake of simplicity, we will be preparing we will be setting one reaction of 5 micrograms per ml in which already I have added 950 micro liters of water.

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Then from the stock concentration of tyrosine that is 0.2 mg per ml, we will be adding 50 micro liters of tyrosine. Then for setting the reaction sodium carbonate and folins reagent is to be added. Sodium carbonated carbonate is added to maintain pH which be which can be changed due to this folins reagent. So, now we will be adding 1 ml of sodium carbonate and 100 microliters of folins reagent.

Ok, now as we have set the reactions for different concentrations of tyrosine now, we will incubate it at 37 degree Celsius for 30 minutes. We have prepared different concentrations of casein as we have prepared for L tyrosine. Casein is prepared with the help of stock solution 6.5 mg per ml which is prepared in potassium phosphate buffer. Different concentrations of caseins have been prepared from 0 mg per ml to 6.5 mg per ml.

Now, for setting reaction in 6.5 mg per ml, we will be adding trypsin that is 100 micro liters of trypsin. Same amount of trypsin is added in all the eppendorfs and now, all these eppendorfs are kept for incubation for 30 minutes at 37 degree Celsius. After incubation of 30 minutes at 37 degree Celsius, now 500 microliters of trichloroacetic acid is added in the reaction.

This trichloroacetic acid is added to stop the reaction of substrate and protease. Now, 100 microliters of enzyme diluent solution is also added in the reaction. This enzyme diluent solution is added to dissolve the solid proteases and now, these all eppendorfs are kept for incubation at 37 degree Celsius for 10 minutes. After incubation of 10 minutes we have performed centrifugation at 10,000 rpm for 10 minutes in order to settle down the pallet obtained due to the protein precipitation caused by trichloroacetic acid.

Now, from this eppendorf we will collect 200 microliters of filtrate in another eppendorf tube. Now, in this tube 500 microliters of sodium carbonate is added as we have discussed previously sodium carbonate is added in order to maintain the pH change caused by folins reagent. Now, 200 microliters of folins reagent is added in all the reactions. Now, all the eppendorfs are kept for incubation at 37 degree Celsius for 30 minutes in order to develop the blue color chromophores.

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After incubating the samples for 30 minutes, we have taken readings in UV visible spectrophotometer at 660 nanometer. The now then we have observed that absorbance

values were increasing with increasing concentration as well as blue color intensity was also increasing with increasing concentrations of both standards as well as casein protease assay samples. Now, in order to in order to determine enzymatic activity of trypsin and K m value we will plot Michaelis-Menten curve which we will be discussing further.

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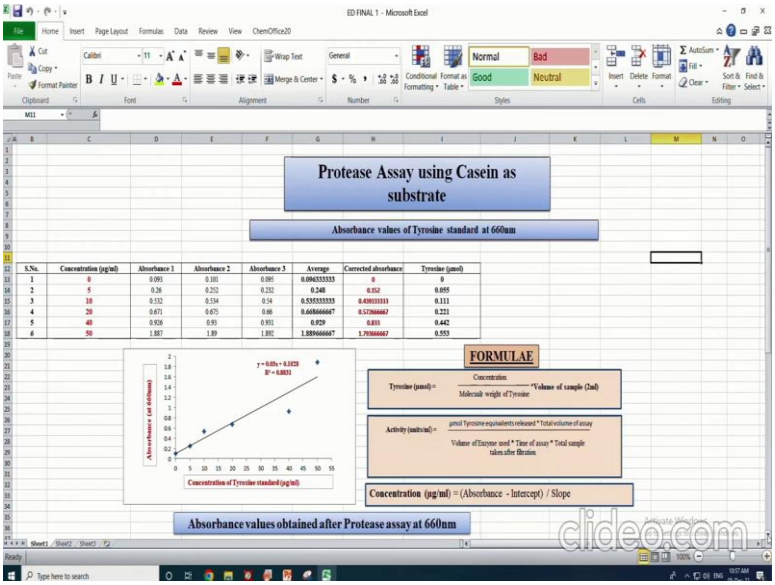


After completing the experiment now we are going to plot calibration curve for tyrosine standards. Here are the different concentrations of L tyrosine from 0 micrograms per ml to 50 microgram per ml. The absorbance value which were taken in triplicates at 660 nanometer in UV visible spectrophotometer are shown here. Firstly, we have calculated average of different absorbance as shown here and corrected absorbance has been calculated by subtracting absorbance of blank that is 0.096 from all other absorbance values.

Then a standard graph is plotted by taking absorbance at y-axis and concentration of tyrosine standard at x-axis. Here we are getting R square of 0.8831. Then amount of tyrosine in micromoles has been calculated by using this formula that is concentration upon molecular weight of tyrosine into volume of sample. Here molecular weight of tyrosine is 181.19 grams per mole.

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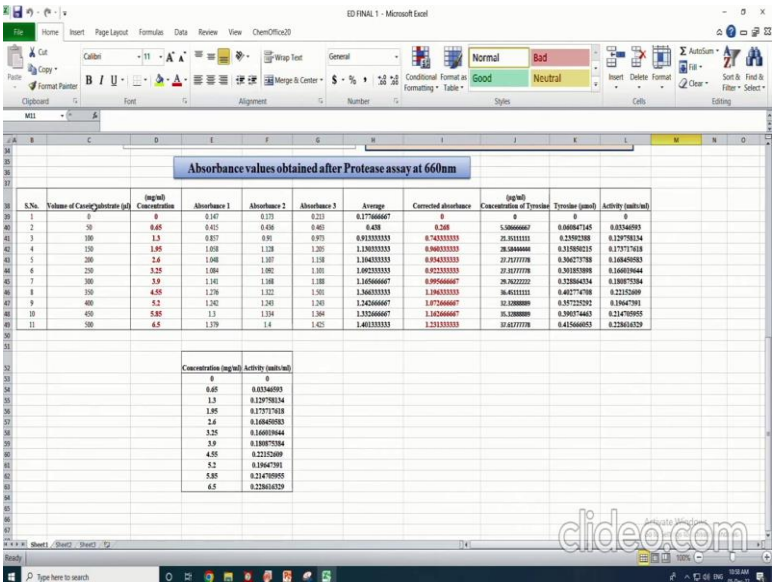


Now, coming to our main experiment that is protease assay between casein and trypsin, here we have taken casein as substrate and trypsin as our protease. As after the completion of reaction tyrosine is liberated from substrate casein, to determine the concentration of liberated tyrosine we have taken absorbance values in triplicates for reaction mixture and calculated the corresponding concentration using standard curve plotted before.

The amount of tyrosine is calculated in terms of micromoles the amount of tyrosine this tyrosine is calculated in terms of micromoles by using this formula and activity of enzyme that is trypsin in units per ml has been calculated by using the formula, that is, micromoles tyrosine equivalents released into total volume of assay upon volume of enzyme used into time of assay into total sample taken after filtration.

Here total volume of assay was 1.1 ml volume of enzyme used was 100 microliters; time of assay was 30 minutes and total sample taken after filtration or centrifugation was 200 microliters. Now, to plot Michaelis-Menten curve all the required parameters are summarized here. So, I am going to copy these values from here.

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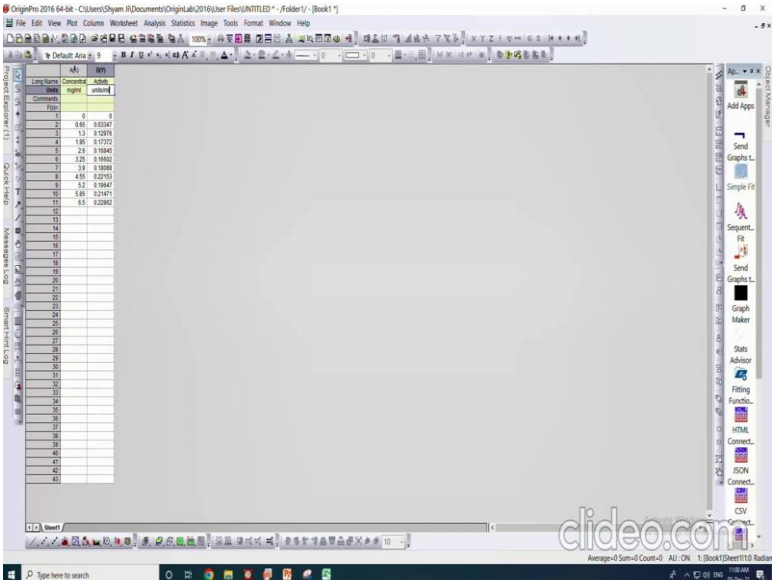

And, we will paste these values in the Origin software here we will paste these values and we will label x-axis as concentration, Concentration in mg per ml and we will label y-axis as activity, Activity in units per ml. Now, we will plot the scatter graph from here.

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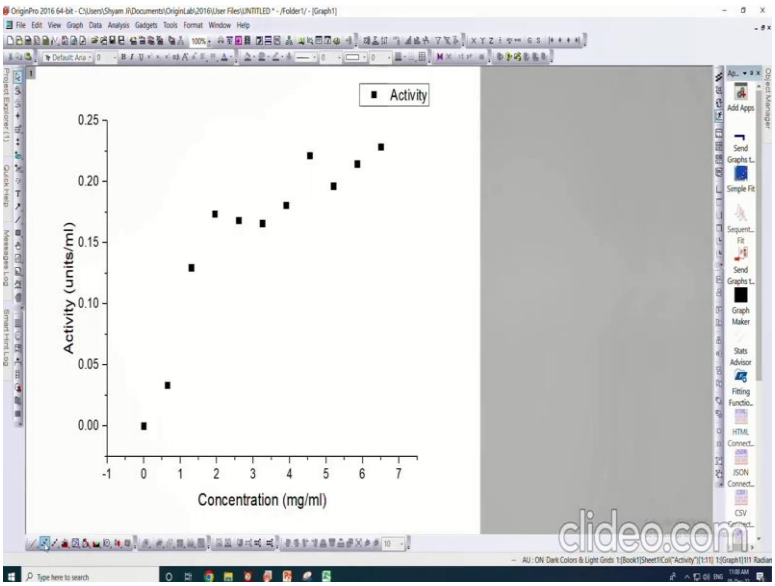


Here is this scatter graph. Firstly, scale can be corrected.

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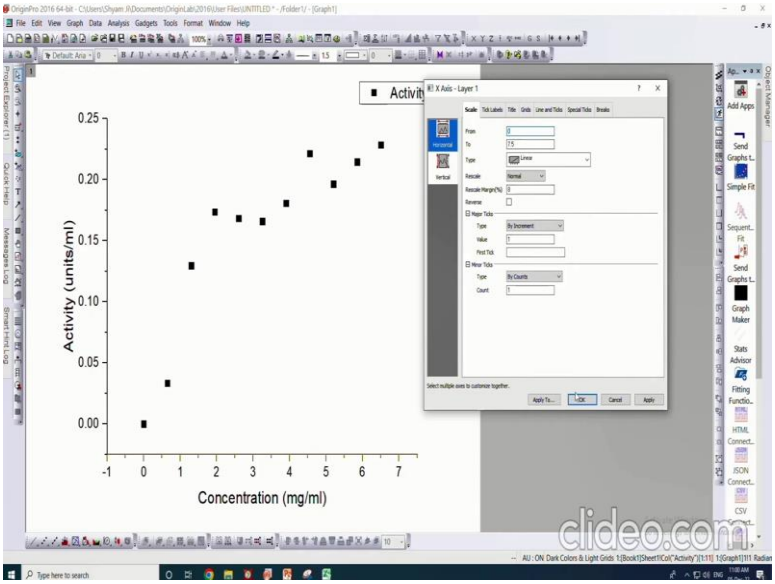


Before analysis of the graph, we will make it 0 we will do the same with y-axis also, ok.

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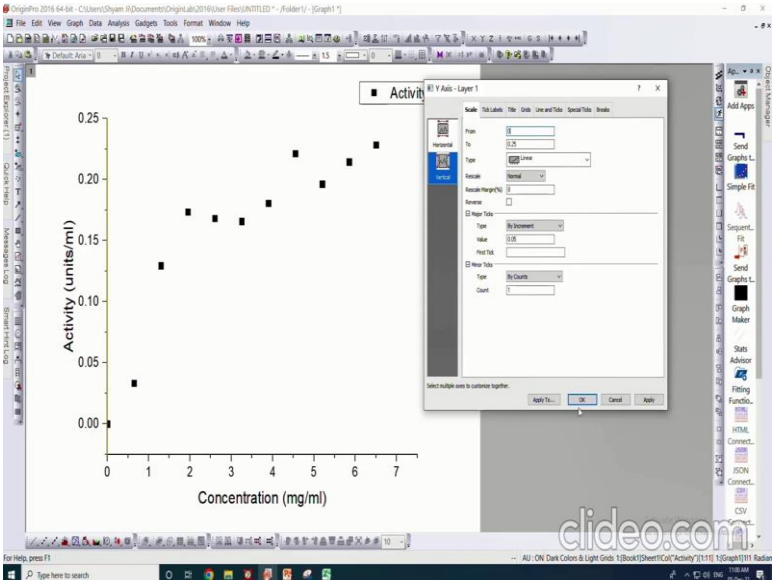

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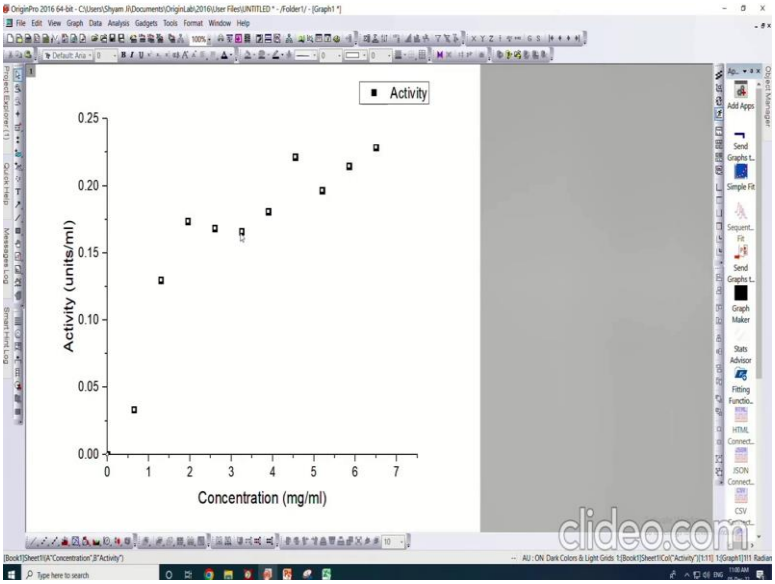


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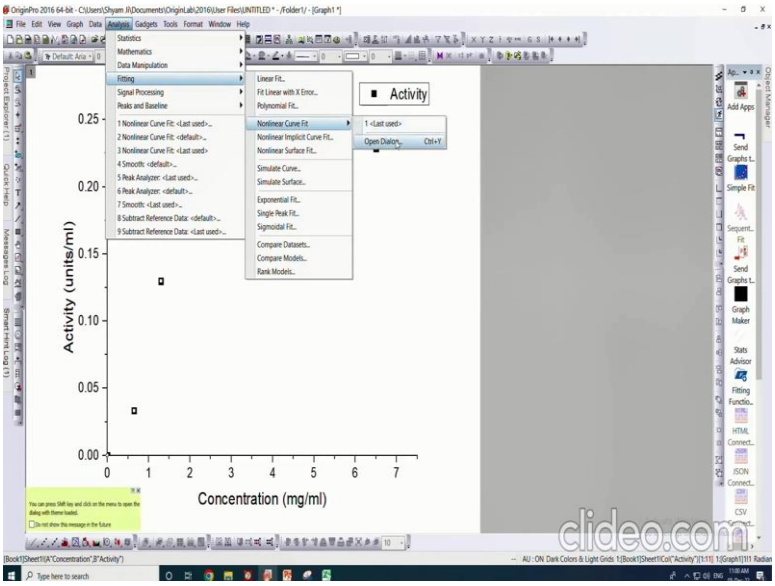


Now, for the analysis we will go to Analysis, Fitting, Nonlinear Curve Fit. A dialogue box will open.

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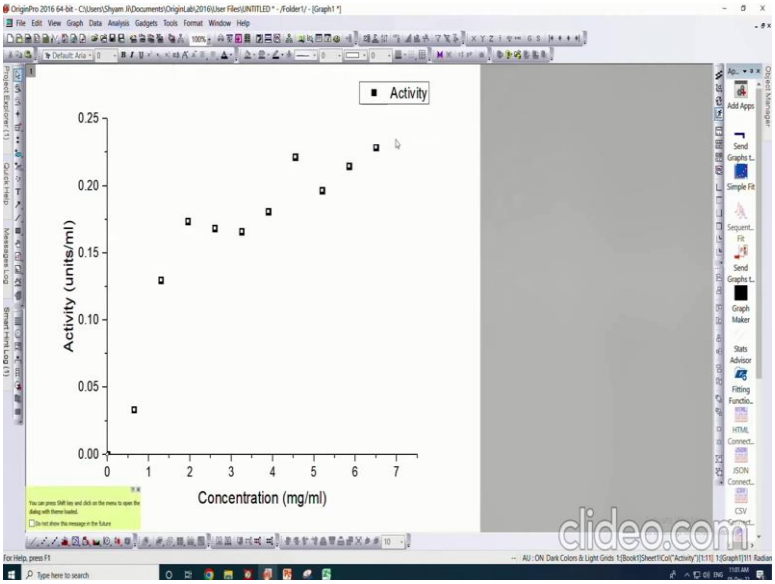


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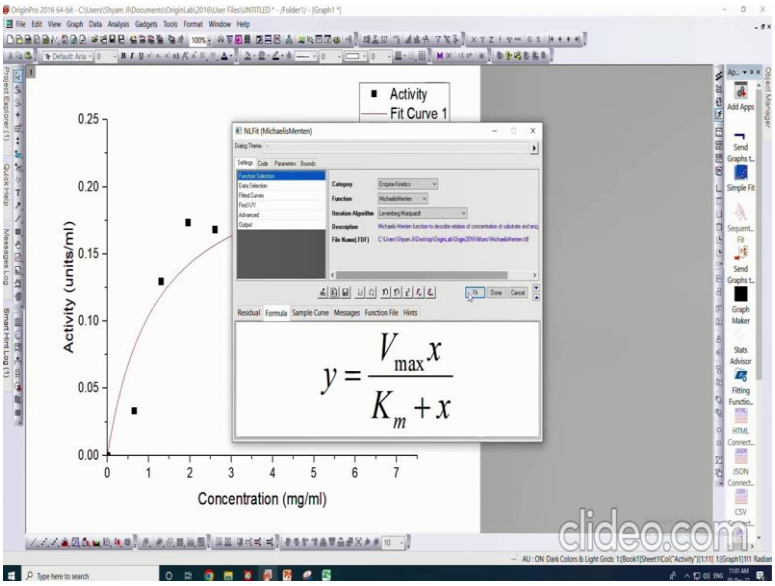


In which we have to set parameters like Category we have to set Enzyme Kinetics; Function we have to set Michaelis-Menten. This is the Michaelis- Menten equation. This is the formula for Michaelis- Menten equation that is y equal to V max x upon K m plus x here y is the activity of enzyme.

V max is the maximum activity of enzyme x stands for the substrate which is casein. In this case K m is the Michaelis-Menten constant which denotes the affinity of enzyme for

its substrate. Theoretically, K m is the concentration of substrate at which enzyme activity is half of maximum.

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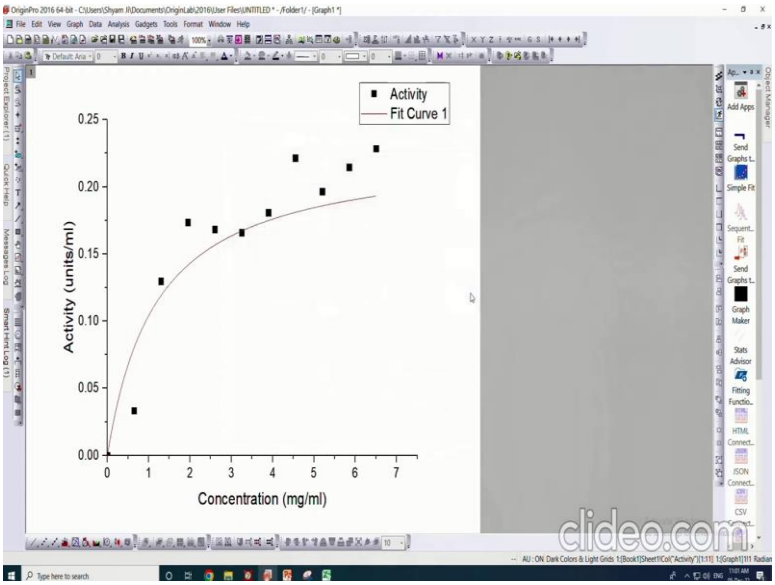


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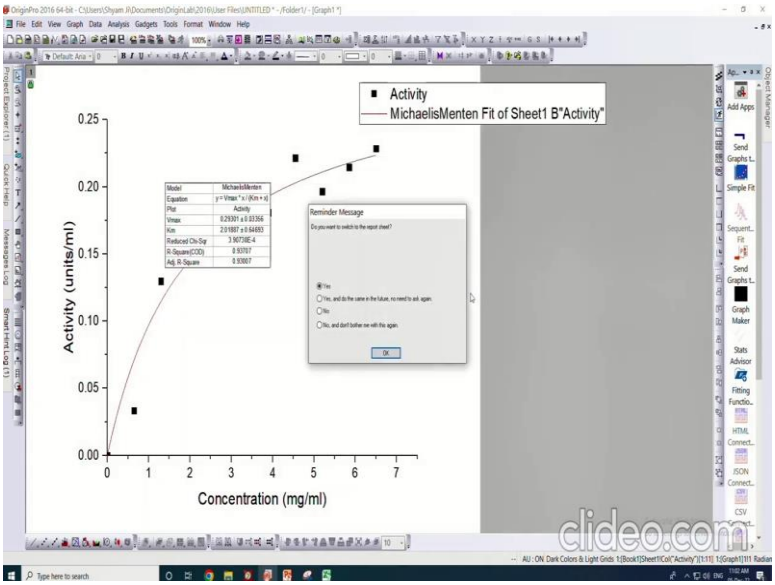


Now, we will Fit this. This dialogue box shows the V max value of 0.2 and K m of 2.0.

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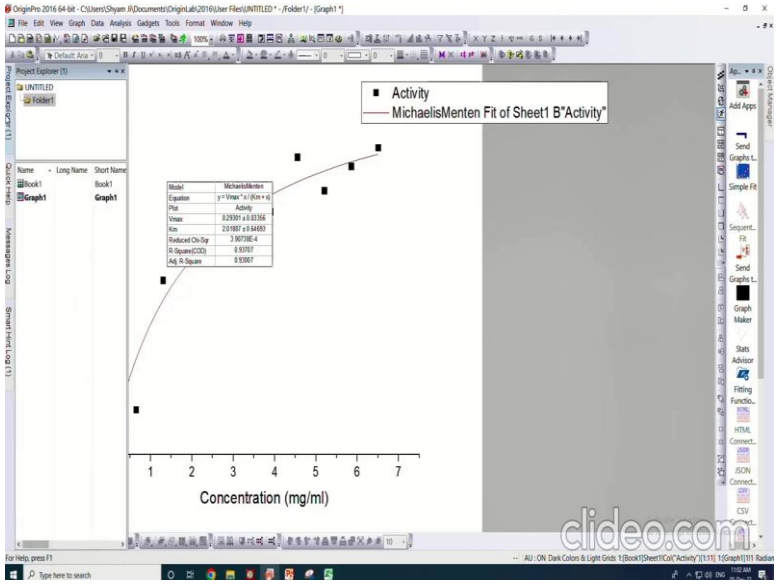


Now, we will open another dialogue box by clicking this Book1, ok.

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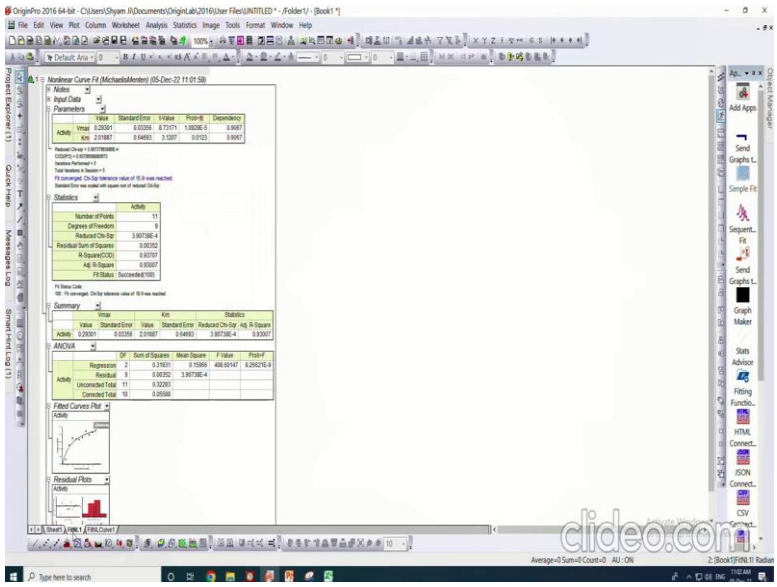

This dialogue box tells us about accuracy of the curve fitting. From here we can say that Fit Status is 100 percent Succeeded that is and R square is 0.93 and as we have seen before K m is 2.0 and V max is 0.2. So, by the help of this protease assay we concluded that this protocol will enable enzyme activity of any unknown protease. In addition, this assay is useful for ensuring that proteases have precisely determined activity before using them for further experiments. Thank you.

So, with this I would like to conclude my lecture here. In our in this particular lecture we have discussed about the different aspect of the enzyme kinetics. We have discussed about the Michaelis-Menten approach and we have also discussed about the Briggs-Haldane approach, how you can be able to express the consideration of the enzyme substrate complex and how you can be able to use that for deriving the different types of kinetic parameters.

At the end we have also discussed about how you can be able to determine the K m values from the given data and how you can be able to use and what will be the significance of the K m in the enzyme kinetics. So, with this I would like to conclude my lecture here. In our subsequent lecture, we are going to discuss some more aspects of the enzymes in this particular course.

Thank you.