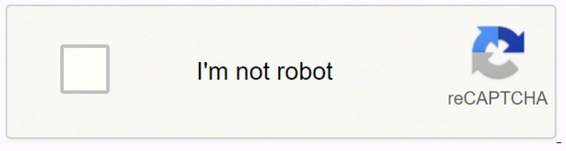

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What types of food production processes use enzymes

Enzyme kinetics is the study of factors that determine the speed of enzyme-catalysed reactions. It utilizes some mathematical equations that can be confusing to students when they first encounter them. However, the theory of kinetics is both logical and simple, and it is essential to develop an understanding of this subject in order to be able to appreciate the role of enzymes both in metabolism and in biotechnology.Assays (measurements) of enzyme activity can be performed in either a discontinuous or continuous fashion. Discontinuous methods involve mixing the substrate and enzyme together and measuring the product formed after a set period of time, so these methods are generally easy and quick to perform. In general we would use such discontinuous assays when we know little about the system (and are making preliminary investigations), or alternatively when we know a great deal about the system and are certain that the time interval we are choosing is appropriate.In continuous enzyme assays we would generally study the rate of an enzyme-catalysed reaction by mixing the enzyme with the substrate and continuously measuring the appearance of product over time. Of course we could equally well measure the rate of the reaction by measuring the disappearance of substrate over time. Apart from the actual direction (one increasing and one decreasing), the two values would be identical. In enzyme kinetics experiments, for convenience we very often use an artificial substrate called a chromogen that yields a brightly coloured product, making the reaction easy to follow using a colorimeter or a spectrophotometer. However, we could in fact use any available analytical equipment that has the capacity to measure the concentration of either the product or the substrate. In almost all cases we would also add a buffer solution to the mixture. As we shall see, enzyme activity is strongly influenced by pH, so it is important to set the pH at a specific value and keep it constant throughout the experiment.Our first enzyme kinetics experiment may therefore involve mixing a substrate solution (chromogen) with a buffer solution and adding the enzyme. This mixture would then be placed in a spectrophotometer and the appearance of the coloured product would be measured. This would enable us to follow a rapid reaction which, after a few seconds or minutes, might start to slow down, as shown in Figure 4.A common reason for this slowing down of the speed (rate) of the reaction is that the substrate within the mixture is being used up and thus becoming limiting. Alternatively, it may be that the enzyme is unstable and is denaturing over the course of the experiment, or it could be that the pH of the mixture is changing, as many reactions either consume or release protons. For these reasons, when we are asked to specify the rate of a reaction we do so early on, as soon as the enzyme has been added, and when none of the above-mentioned limitations apply. We refer to this initial rapid rate as the initial velocity (v0). Measurement of the reaction rate at this early stage is also quite straightforward, as the rate is effectively linear, so we can simply draw a straight line and measure the gradient (by dividing the concentration change by the time interval) in order to evaluate the reaction rate over this period.We may now perform a range of similar enzyme assays to evaluate how the initial velocity changes when the substrate or enzyme concentration is altered, or when the pH is changed. These studies will help us to characterize the properties of the enzyme under study.The relationship between enzyme concentration and the rate of the reaction is usually a simple one. If we repeat the experiment just described, but add 10% more enzyme, the reaction will be 10% faster, and if we double the enzyme concentration the reaction will proceed twice as fast. Thus there is a simple linear relationship between the reaction rate and the amount of enzyme available to catalyse the reaction (Figure 5).This relationship applies both to enzymes in vivo and to those used in biotechnological applications, where regulation of the amount of enzyme present may control reaction rates.When we perform a series of enzyme assays using the same enzyme concentration, but with a range of different substrate concentrations, a slightly more complex relationship emerges, as shown in Figure 6. Initially, when the substrate concentration is increased, the rate of reaction increases considerably. However, as the substrate concentration is increased further the effects on the reaction rate start to decline, until a stage is reached where increasing the substrate concentration has little further effect on the reaction rate. At this point the enzyme is considered to be coming close to saturation with substrate, and demonstrating its maximal velocity (Vmax). Note that this maximal velocity is in fact a theoretical limit that will not be truly achieved in any experiment, although we might come very close to it.The relationship described here is a fairly common one, which a mathematician would immediately identify as a rectangular hyperbola. The equation that describes such a relationship is as follows: The two constants a and b thus allow us to describe this hyperbolic relationship, just as with a linear relationship (y = mx + c), which can be expressed by the two constants m (the slope) and c (the intercept).We have in fact already defined the constant a — it is Vmax. The constant b is a little more complex, as it is the value on the x-axis that gives half of the maximal value of y. In enzymology we refer to this as the Michaelis constant (Km), which is defined as the substrate concentration that gives half-maximal velocity.Our final equation, usually called the Michaelis–Menten equation, therefore becomes: Initial rate of reaction(v0)=Vmax×Substrate concentrationSubstrate concentration+KmIn 1913, Leonor Michaelis and Maud Menten first showed that it was in fact possible to derive this equation mathematically from first principles, with some simple assumptions about the way in which an enzyme reacts with a substrate to form a product. Central to their derivation is the concept that the reaction takes place via the formation of an ES complex which, once formed, can either dissociate (productively) to release product, or else dissociate in the reverse direction without any formation of product. Thus the reaction can be represented as follows, with k1, k−1 and k2 being the rate constants of the three individual reaction steps: The Michaelis–Menten derivation requires two important assumptions. The first assumption is that we are considering the initial velocity of the reaction (v0), when the product concentration will be negligibly small (i.e. [S] ≫ [P]), such that we can ignore the possibility of any product reverting to substrate. The second assumption is that the concentration of substrate greatly exceeds the concentration of enzyme (i.e. [S]≫[E]).The derivation begins with an equation for the expression of the initial rate, the rate of formation of product, as the rate at which the ES complex dissociates to form product. This is based upon the rate constant k2 and the concentration of the ES complex, as follows: Since ES is an intermediate, its concentration is unknown, but we can express it in terms of known values. In a steady-state approximation we can assume that although the concentration of substrate and product changes, the concentration of the ES complex itself remains constant. The rate of formation of the ES complex and the rate of its breakdown must therefore balance, where: Rate of ES complex formation = k1[E][S] and Rate of ES complex breakdown = (k−1 + k2)[ES]Hence, at steady state: This equation can be rearranged to yield [ES] as follows: The Michaelis constant Km can be defined as follows: Equation 2 may thus be simplified to: Since the concentration of substrate greatly exceeds the concentration of enzyme (i.e. [S] ≫ [E]), the concentration of uncombined substrate [S] is almost equal to the total concentration of substrate. The concentration of uncombined enzyme [E] is equal to the total enzyme concentration [E]T minus that combined with substrate [ES]. Introducing these terms to Equation 3 and solving for ES gives us the following: We can then introduce this term into Equation 1 to give: The term k2[E]T in fact represents Vmax, the maximal velocity. Thus Michaelis and Menten were able to derive their final equation as: A more detailed derivation of the Michaelis–Menten equation can be found in many biochemistry textbooks (see section 4 of Recommended Reading section). There are also some very helpful web-based tutorials available on the subject.Michaelis constants have been determined for many commonly used enzymes, and are typically in the lower millimolar range (Table 5).Typical range of values of the Michaelis constant.EnzymeKm (mmol l−1)Carbonic anhydrase26Chymotrypsin15Ribonuclease8Tyrosyl-tRNA synthetase0.9Pepsin0.3It should be noted that enzymes which catalyse the same reaction, but which are derived from different organisms, can have widely differing Km values. Furthermore, an enzyme with multiple substrates can have quite different Km values for each substrate.A low Km value indicates that the enzyme requires only a small amount of substrate in order to become saturated. Therefore the maximum velocity is reached at relatively low substrate concentrations. A high Km value indicates the need for high substrate concentrations in order to achieve maximum reaction velocity. Thus we generally refer to Km as a measure of the affinity of the enzyme for its substrate—in fact it is an inverse measure, where a high Km indicates a low affinity, and vice versa.The Km value tells us several important things about a particular enzyme. An enzyme with a low Km value relative to the physiological concentration of substrate will probably always be saturated with substrate, and will therefore act at a constant rate, regardless of variations in the concentration of substrate within the physiological range.An enzyme with a high Km value relative to the physiological concentration of substrate will not be saturated with substrate, and its activity will therefore vary according to the concentration of substrate, so the rate of formation of product will depend on the availability of substrate.If an enzyme acts on several substrates, the substrate with the lowest Km value is frequently assumed to be that enzyme's ‘natural’ substrate, although this may not be true in all cases.If two enzymes (with similar Vmax) in different metabolic pathways compete for the same substrate, then if we know the Km values for the two enzymes we can predict the relative activity of the two pathways. Essentially the pathway that has the enzyme with the lower Km value is likely to be the ‘preferred pathway’, and more substrate will flow through that pathway under most conditions. For example, phosphofructokinase (PFK) is the enzyme that catalyses the first committed step in the glycolytic pathway, which generates energy in the form of ATP for the cell, whereas glucose-1-phosphate uridylyltransferase (GUT) is an enzyme early in the pathway leading to the synthesis of glycogen (an energy storage molecule). Both enzymes use hexose monophosphates as substrates, but the Km of PFK for its substrate is lower than that of GUT for its substrate. Thus at lower cellular hexose phosphate concentrations, PFK will be active and GUT will be largely inactive. At higher hexose phosphate concentrations both pathways will be active. This means that the cells only store glycogen in times of plenty, and always give preference to the pathway of ATP production, which is the more essential function. Very often it is not possible to estimate Km values from a direct plot of velocity against substrate concentration (as shown in Figure 6) because we have not used high enough substrate concentrations to come even close to estimating maximal velocity, and therefore we cannot evaluate half-maximal velocity and thus Km. Fortunately, we can plot our experimental data in a slightly different way in order to obtain these values. The most commonly used alternative is the Lineweaver–Burk plot (often called the double-reciprocal plot). This plot linearizes the hyperbolic curved relationship, and the line produced is easy to extrapolate, allowing evaluation of Vmax and Km. For example, if we obtained only the first seven data points in Figure 6, we would have difficulty estimating Vmax from a direct plot as shown in Figure 7a.However, as shown in Figure 7b, if these seven points are plotted on a graph of 1/velocity against 1/substrate concentration (i.e. a double-reciprocal plot), the data are linearized, and the line can be easily extrapolated to the left to provide intercepts on both the y-axis and the x-axis, from which Vmax and Km, respectively, can be evaluated.One significant practical drawback of using the Lineweaver–Burk plot is the excessive influence that it gives to measurements made at the lowest substrate concentrations. These concentrations might well be the most prone to error (due to difficulties in making multiple dilutions), and result in reaction rates that, because they are slow, might also be most prone to measurement error. Often, as shown in Figure 8, such points when transformed on the Lineweaver–Burk plot have a significant impact on the line of best fit estimated from the data, and therefore on the extrapolated values of both Vmax and Km. The two sets of points shown in Figure 8 are identical except for the single point at the top right, which reflects (because of the plot's double-reciprocal nature) a single point derived from a very low substrate concentration and a low reaction rate. However, this single point can have an enormous impact on the line of best fit and the accompanying estimates of kinetic constants.In fact there are other kinetic plots that can be used, including the Eadie–Hofstee plot, the Hanes plot and the Eisenthal–Cornish-Bowden plot, which are less prone to such problems. However, the Lineweaver–Burk plot is still the most commonly described kinetic plot in the majority of enzymology textbooks, and thus retains its influence in undergraduate education.Various environmental factors are able to affect the rate of enzyme-catalysed reactions through reversible or irreversible changes in the protein structure. The effects of pH and temperature are generally well understood.Most enzymes have a characteristic optimum pH at which the velocity of the catalysed reaction is maximal, and above and below which the velocity declines (Figure 9).The pH profile of β-glucosidase.The pH profile is dependent on a number of factors. As the pH changes, the ionization of groups both at the enzyme's active site and on the substrate can alter, influencing the rate of binding of the substrate to the active site. These effects are often reversible. For example, if we take an enzyme with an optimal pH (pHopt) of 7.0 and place it in an environment at pH 6.0 or 8.0, the charge properties of the enzyme and the substrate may be suboptimal, such that binding and hence the reaction rate are lowered. If we then readjust the pH to 7.0, the optimal charge properties and hence the maximal activity of the enzyme are often restored. However, if we place the enzyme in a more extreme acidic or alkaline environment (e.g. at pH 1 or 14), although these conditions may not actually lead to changes in the very stable covalent structure of the protein (i.e. its configuration), they may well produce changes in the conformation (shape) of the protein such that, when it is returned to pH 7.0, the original conformation and hence the enzyme's full catalytic activity are not restored.It should be noted that the optimum pH of an enzyme may not be identical to that of its normal intracellular surroundings. This indicates that the local pH can exert a controlling influence on enzyme activity.The effects of temperature on enzyme activity are quite complex, and can be regarded as two forces acting simultaneously but in opposite directions. As the temperature is raised, the rate of molecular movement and hence the rate of reaction increases, but at the same time there is a progressive inactivation caused by denaturation of the enzyme protein. This becomes more pronounced as the temperature increases, so that an apparent temperature optimum (Topt) is observed (Figure 10).Thermal denaturation is time dependent, and for an enzyme the term ‘optimum temperature’ has little real meaning unless the duration of exposure to that temperature is recorded. The thermal stability of an enzyme can be determined by first exposing the protein to a range of temperatures for a fixed period of time, and subsequently measuring its activity at one favourable temperature (e.g. 25°C).The temperature at which denaturation becomes important varies from one enzyme to another. Normally it is negligible below 30°C, and starts to become appreciable above 40°C. Typically, enzymes derived from microbial sources show much higher thermal stability than do those from mammalian sources, and enzymes derived from extremely thermophilic microorganisms, such as thermolysin (a protease from Bacillus thermoproteolyticus) and Taq polymerase (a DNA polymerase from Thermus aquaticus), might be completely thermostable at 70°C and still retain substantial levels of activity even at 100°C.Having spent time learning about enzyme kinetics and the Michaelis–Menten relationship, it is often quite disconcerting to find that some of the most important enzymes do not in fact display such properties. Allosteric enzymes are key regulatory enzymes that control the activities of metabolic pathways by responding to inhibitors and activators. These enzymes in fact show a sigmoidal (S-shaped) relationship between reaction rate and substrate concentration (Figure 11), rather than the usual hyperbolic relationship. Thus for allosteric enzymes there is an area where activity is lower than that of an equivalent ‘normal’ enzyme, and also an area where activity is higher than that of an equivalent ‘normal’ enzyme, with a rapid transition between these two phases. This is rather like a switch that can quickly be changed from ‘off’ (low activity) to ‘on’ (full activity).Most allosteric enzymes are polymeric—that is, they are composed of at least two (and often many more) individual polypeptide chains. They also have multiple active sites where the substrate can bind. Much of our understanding of the function of allosteric enzymes comes from studies of haemoglobin which, although it is not an enzyme, binds oxygen in a similarly co-operative way and thus also demonstrates this sigmoidal relationship. Allosteric enzymes have an initially low affinity for the substrate, but when a single substrate molecule binds, this may break some bonds within the enzyme and thereby change the shape of the protein such that the remaining active sites are able to bind with a higher affinity. Therefore allosteric enzymes are often described as moving from a tensed state or T-state (low affinity) in which no substrate is bound, to a relaxed state or R-state (high affinity) as substrate binds. Other molecules can also bind to allosteric enzymes, at additional regulatory sites (i.e. not at the active site). Molecules that stabilize the protein in its T-state therefore act as allosteric inhibitors, whereas molecules that move the protein to its R-state will act as allosteric activators or promoters.A good example of an allosteric enzyme is aspartate transcarbamoylase (ATCase), a key regulatory enzyme that catalyses the first committed step in the sequence of reactions that produce the pyrimidine nucleotides which are essential components of DNA and RNA. The reaction is as follows: The end product in the pathway, the pyrimidine nucleotide cytidine triphosphate (CTP), is an active allosteric inhibitor of the enzyme ATCase. Therefore when there is a high concentration of CTP in the cell, this feeds back and inhibits the ATCase enzyme, reducing its activity and thus lowering the rate of production of further pyrimidine nucleotides. As the concentration of CTP in the cell decreases then so does the inhibition of ATCase, and the resulting increase in enzyme activity leads to the production of more pyrimidine nucleotides. This negative feedback inhibition is an important element of biochemical homeostasis within the cell. However, in order to synthesize DNA and RNA, the cell requires not only pyrimidine nucleotides but also purine nucleotides, and these are needed in roughly equal proportions. Purine synthesis occurs through a different pathway, but interestingly the final product, the purine nucleotide adenosine triphosphate (ATP), is a potent activator of the enzyme ATCase. This is logical, since when the cell contains high concentrations of purine nucleotides it will require equally high concentrations of pyrimidine nucleotides in order for these two types of nucleotide to combine to form the polymers DNA and RNA. Thus ATCase is able to regulate the production of pyrimidine nucleotides within the cell according to cellular demand, and also to ensure that pyrimidine nucleotide synthesis is synchronized with purine nucleotide synthesis—an elegant biochemical mechanism for the regulation of an extremely important metabolic process.There are some rare, although important, cases of monomeric enzymes that have only one substrate-binding site but are capable of demonstrating the sigmoidal reaction kinetics characteristic of allosteric enzymes. Particularly noteworthy in this context is the monomeric enzyme glucokinase (also called hexokinase IV), which catalyses the phosphorylation of glucose to glucose-6-phosphate (which may then either be metabolized by the glycolytic pathway or be used in glycogen synthesis). It has been postulated that this kinetic behaviour is a result of individual glucokinase molecules existing in one of two forms—a low-affinity form and a high-affinity form. The low-affinity form of the enzyme reacts with its substrate (glucose), is then turned into the high-affinity form, and remains in that state for a short time before slowly returning to its original low-affinity form (demonstrating a so-called slow transition). Therefore at high substrate concentrations the enzyme is likely to react with a second substrate molecule soon after the first one (i.e. while still in its high-affinity form), whereas at lower substrate concentrations the enzyme may transition back to its low-affinity form before it reacts with subsequent substrate molecules. This results in its characteristic sigmoidal reaction kinetics.

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